THE ROLE OF GEOGRAPHY IN THE EVOLUTION OF GAMETE INCOMPATIBILITY IN HYBRIDIZING BLUE MUSSELS

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ABSTRACT

The examination of natural hybridization and hybrid zones are useful tools to examine the evolution of prezygotic and post zygotic mechanisms through which reproductive isolation develops in marine environments that typically lack the absolute physical barriers that are requisite for traditional allopatric models of genetic differentiation thought to lead to species formation. For blue mussels species that readily hybridize in areas of sympatry, post zygotic mechanisms have been the focus of the majority of investigations addressing isolating mechanisms. However, for free-spawning marine invertebrates, gametic incompatibility can facilitate the evolution of "complete" reproductive isolation in sympatric species through the strengthening of prezygotic isolating traits as a result of selection against hybrids and hybridization (i.e. reinforcement).

The reinforcement of pre-mating isolation, as evidenced by a pattern of reproductive character displacement, was investigated in the hybridizing blue mussels, *Mytilus edulis* and *M. trossulus* within the Gulf of Maine. Using *in vitro* fertilization experiments, a simple comparison was made evaluating levels of heterospecific gamete compatibility, in allopatric, *M. edulis* females, compared to *M. edulis* females from a sympatric, hybridizing, population. Partial compatibility of *M. edulis* females in heterospecific crosses was observed in both sympatric and allopatric populations, however in a pattern opposite to that expected under a theory of reinforcement. *Mytilus edulis* females from allopatric populations were more strongly blocked to heterospecific fertilization than *M. edulis* females from sympatric populations.

The absence of a signal of reproductive character displacement consistent with the process of reinforcement suggests that the "atypically" compatible female found in sympatric populations may be a product of introgression, with highly introgressed individuals undetected at

the current level of resolution available. The absence of reproductive character displacement should not, however, eliminate the role that reinforcement may play in the pattern of interbreeding, and non-fusion, in these hybridizing species. A comparison of patterns in heterospecific gamete incompatibility between western and northeastern Atlantic hybrid zones may prove to be valuable for studying the process of reinforcement, as well as lead to a greater understanding of the role of hybridization in species formation.

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My special thanks are extended to all who hand-held my neurosis, including the Pawlik Lab ("Helping-Hand Lab"), Russ Peterson, Enrico Tronchin (in the 11th hour) and especially (and always) D. Wilson Freshwater. I would also like to thank Greg Dietl for his support, advice, and for introducing me to the next "shiny-object."

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DEDICATION

This thesis is dedicated to my son, Jake, the contents of which he could care less – and for that perspective I will be forever grateful. To him I say, thank you for playing Ishmael to my Ahab, and promise our next voyage will sail to the west.

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INTRODUCTION

Marine hybridization and hybrid zones

Natural hybridization and hybrid zones are of particular interest to evolutionary biologists because they provide the opportunity to address the process of speciation, via an examination of the evolution of prezygotic and postzygotic mechanisms through which reproductive isolation develops. A natural hybrid zone occurs when individuals from genetically distinct species meet and successfully interbreed. This creates an area where individuals are of mixed lineage, and surrounded by populations of unmixed lineage (Barton and Hewitt 1985; Harrison 1993; Arnold 1997; Gardner 1997). Here the definition of the term hybrid is not restricted to first generation crosses (F_1) resulting from the interbreeding of genetically distinct parents, but includes later generation backcrosses.

The examination of hybridization and hybrid zones are useful tools to address questions of how reproductive isolation evolves in marine environments that lack the absolute physical barriers that are requisite for traditional allopatric models of genetic differentiation (i.e. divergence via drift and natural selection, Mayr 1942) thought to lead to species formation (Palumbi 1992, 1994; Gardner 1997). While hybridization between marine species was previously thought to be rare, a review by Gardner (1997) indicates that "it is not an uncommon phenomenon", providing 108 documented cases of hybridization and 34 hybrid zones across both algal and animal taxa. The bulk of these cases are observations of one or more hybrid individuals produced from the interbreeding of genetically distinct parents, or isolated instances of observed hybridization, as opposed to geographically structured patters of hybridization as are found in hybrid zones. Isolated instances of hybridization notwithstanding, for marine taxa, in

general, there appears to be a bias in documentation of `hybridization frequency, and formation of hybrid zones, toward that of marine invertebrate species.

Gamete incompatibility, hybridization, and reinforcement

The tendency for marine invertebrates to hybridize may be a consequence of the prevalence of broadcast spawning as a method of reproduction (Gardner 1997). Broadcast spawning, or free-spawning, involves the release of gametes into directly into the water column for fertilization, with little control over the fate of those gametes. In the absence of behavioral traits that ensure fertilization by a conspecific, free-spawning marine invertebrates can maintain species integrity through gamete specificity, or incompatibility, which results in the failure of sperm from one species to fertilize the egg of another species.

Fertilization can be easily studied in free-spawning marine invertebrates, and has been the focus of gamete incompatibility studies in a variety of marine taxa (e.g. corals; Knowlton et al. 1997, polychaetes: Pawlik 1988, Pernet 1999; sea stars: Byrne and Anderson 1994; abalone: Leighton and Lewis 1982; urchins: Lessios and Cunningham 1990; Palumbi and Metz 1991; McCartney and Lessios 2002). Among these, the mechanisms of gamete interactions and fertilization are particularly well known for urchins and abalone (Palumbi 1999; Vacquier 1998). For example, in urchins the block to heterospecific fertilization occurs before plasma membrane fusion, but after sperm penetration of egg jelly coat, attachment, and penetration of the egg vitelline membrane (Palumbi 1992). This block relies on species-specific variation in a sperm protein, bindin, that facilitates attachment to the egg, consequently heterospecific sperm fail to completely attach to the egg (Palumbi 1999). For abalone the block to hybridization involves another sperm protein, lysin, that is believed to exhibit species specificity in its ability to dissolve

a hole in the egg envelope (Vacquier 1998; Kresge et al. 2001). The evolution of species-specific fertilization may be an important part of the process by which new species arise in sympatric populations of closely related free-spawning marine invertebrates (Kresge et al. 2001). Equally, the degree of gametic incompatibility due to the evolution of these rapidly evolving proteins can impact the patterns and direction of hybridization observed in free-spawning marine invertebrates, as well as facilitate the evolution of "complete" reproductive isolation in sympatric species following secondary contact.

When a barrier to gene flow between populations, such as gamete incompatibility, is incomplete contact between closely related species can lead to hybridization. The evolution of incomplete barriers may be due to a relatively short amount of time since species separation, or when natural selection is not sufficiently strong within an isolated population. Reinforcement theory describes a process where prezygotic isolating traits are strengthened following secondary contact between two closely related species that have not yet achieved reproductive isolation in allopatry, through selection against hybrids (Dobzhansky 1937; Butlin 1989; Liou and Price 1994). A common method used to investigate the reinforcement of pre-mating isolation is to evaluate the species for evidence of reproductive character displacement (RCD) – differences in reproductively isolating traits exhibited by populations of the same species that can be attributed to the presence of a second species when found in sympatry as compared to those traits displayed in allopatry (Blair 1964; Howard 1993).

It is not unlikely that a pattern of RCD could be observed in free-spawning marine invertebrates, and with the reproductive behavior reduced to gamete interactions, addressed using either *in vitro* fertilization assays to compare patterns of gamete incompatibility between sympatric and allopatric populations or through comparison of reproductive protein sequences.

The criteria for establishing the process of reinforcement requires, among other things, that there be secondary contact of species that have diverged in isolation, with assortative mating selecting against hybridization. Echinoid species used in past studies addressing the evolution of gamete incompatibility fit the above criteria, displaying genetic divergence, occurring in mixed species assemblages, and for the most part displaying patterns of incompatibility in broadly sympatric populations, and partial compatibility when range overlap is small or absent (e.g. Lessios and Cunningham 1990; Minor et al. 1991; Levitan 2002; McCartney and Lessios 2002; Geyer and Palumbi 2003).

However in each case, the taxa investigated are no longer hybridizing, or hybrids formed are rare in natural populations. It follows that for a study of RCD, the absence of naturally formed hybrids limits the conclusions that can be drawn about the process of reinforcement acting in those populations (Howard 1993). Examination of gamete interactions and patterns of RCD in *hybridizing* populations allows for the examination of prezygotic mechanisms that have evolved, or are evolving, to limit hybridization following secondary contact. One such opportunity to do so exists in hybridizing populations of blue mussels.

Blue mussel distribution and hybridization

Blue mussels are free-spawning marine invertebrates found throughout temperate and subpolar regions in both the northern and southern hemispheres (Hilbish et al. 2000; Rawson et al. 2001). There are currently three recognized species of blue mussel that are within what is referred to as the *Mytilus edulis* complex; *Mytilus edulis* (Linnaeus 1758), *Mytilus galloprovincialis* (Lamark 1819) and *Mytilus trossulus* (Gould 1850) (McDonald et al. 1991). Analysis of nuclear and mitochondrial DNA indicate that *Mytilus edulis* and *Mytilus galloprovincialis* are sister taxa, with their divergence beginning about 2 mya (Rawson and

Hilbish 1995, 1998; Quesada et al. 1998, Wilhelm and Hilbish 1998; Hilbish et al. 2000). *Mytilus trossulus* is more distantly related and diverged from the other two species about 3.5 mya (Vermeij 1991; Benyon and Skibinski, 1996; Rawson and Hilbish, 1995, 1998; Hilbish et al. 2000).

In areas where any two of the three species ranges meet and overlap hybrids have been documented (McDonald et al. 1991; Bates and Innes 1995, Gardner 1996; Saavedra et al. 1996; Suchanek et al. 1997; Hilbish et al. 2002). The most extensively researched hybrid zones occur between western European populations of *Mytilus edulis* and *Mytilus galloprovincialis* (Bierne et al. 2002). This hybrid zone extends from southwest England across southwest France and through the Scottish coastline and has a mosaic structure. In this zone environmental factors (e.g. salinity and tidal height/wave exposure) are thought to influence the distribution or hybrids and pure individuals (Coustau et al. 1991; Gardner 1996; Secor at al. 2001; Hilbish et al. 2002). Here, hybridization is common, with hybrid genotypes found at frequencies of 25-80% (Wilhelm and Hilbish 1998). *Mytilus edulis* also readily hybridizes with co-occurring *M. trossulus* in the Baltic Sea (Vainola and Hvilsom 1991). Between these two species in this region, high levels of hybridization and introgression (i.e. the exchange of nuclear or cytoplamic genes between taxa) have led to the suggestion that the two taxa be considered semispecies (Vainola and Hvilsom 1991; Riginos et al. 2002).

Hybridization in the North American *Mytilus* populations is relatively low in comparison to their European counterparts. For example, *Mytilus galloprovincialis* and *M. trossulus* are sympatric along the Pacific coast of North America from the Oregon border to Central California (aprox. 400 km). In this area of overlap, studies have shown hybrid frequencies to be lower thank those of European hybrid zones (7.5-29%) (Suchanek et al. 1997; Rawson et al. 1999).

Introgression has also been shown to be limited and suggests that the hybrids have a reduced fitness relative to the parental genotype (Rawson et al. 1999).

In coastal regions of the northwestern Atlantic, *Mytilus edulis* and *M. trossulus* are sympatric throughout the Canadian Maritimes, and exhibit low levels of hybridization. Samples collected on the east coast of Newfoundland resulted in a bimodally distributed hybrid index indicating little if any introgression (Bates and Innes 1995; Innes and Bates 1999). In Nova Scotia, hybrid frequencies between *Mytilus edulis* and *M. trossulus* were calculated at <2% (Mallet and Carver 1995). However, this low hybridization may not be the rule. Alternate Newfoundland sites exhibited hybrid frequencies of 23-26% (Saavedra et al. 1996; Comesana et al. 1999; Gardner and Thompson 2001). However, the clearest difference in comparison with western European hybrid zones is the near absence of individuals with intermediate hybrid indices in the northwestern Atlantic hybrid zones, even when hybrid frequencies are >20% (Toro et al. 2004).

For blue mussels, postzygotic mechanisms have been the focus of the majority of investigations that seek to understand how *Mytilus* species remain distinct despite hybridization (e.g. Gardner and Thompson 2001; Hilbish et al. 2002). To date, there have been few studies that quantitatively address gametic incompatibly in blue mussels (Rawson et al. 2003 ; Freeman and MacQuarrie 1999; Bierne 2002) yet with the known expression of three lysin-like proteins (Takagi et al. 1994), estimates of trans-artic migrations leading to secondary contact (Vermeij 1991), and geographically expansive hybrid zones; this system is ideal for addressing questions of the evolution of gamete recognition proteins, their role in prezygotic isolation following secondary contact, and patterns of RCD.

Gamete incompatibility in Gulf of Maine blue mussel populations

Within the Gulf of Maine, *Mytilus trossulus* occurs in mixed population with *M. edulis* at frequencies upwards of 50-100% (Rawson et al. 2001). In these areas of sympatry, *M. edulis* and *M. trossulus* have a temporal overlap in gametogenesis and spawning (Maloy et al. 2002) that increases the opportunity for hybridization. However, the 12-13% hybrid frequency documented within these populations (Rawson et al. 2001) is comparable to the low hybridization observed in Canadian populations (Saavedra et al. 1996; Comesana et al. 1999, Gardner and Thompson 2001) and the degree of introgression is similar to that found in hybrid zones involving *Mytilus trossulus* and *M galloprovincialis* on the west coast of North America (Suchanek et al. 1997; Rawson et al. 1999). These findings suggest that stronger barriers to hybridization may exist in these populations.

Equally, the hybrid zone within the Gulf of Maine is bimodal, with a large number of parental genotypes relative to hybrid genotypes (Rawson et al. 2001). For hybridizing species, this bimodality suggests that two important evolutionary processes are at work, (1) selection against hybrids and (2) strong assortative mating or assortative fertilization within hybrid populations (Jiggens and Mallet 2000) resulting in the maintenance of species boundaries between *M. edulis* and *M. trossulus*. Addressing gamete incompatibility, Rawson et al. (2003) found from *in vitro* fertilization assays that for the most part, *Mytilus edulis* and *M. trossulus* behave like "good species," with high levels of conspecific fertilization and low levels of heterospecific fertilization, even when heterospecific sperm concentrations were tested.

Some *M. edulis* females (40%), however, displayed equally high levels of fertilization when crossed with sperm from either their own species male, or sperm from *M. trossulus* males. In crosses between *M. edulis* and *M. trossulus* this asymmetric fertilization appears to be a

female effect (Rawson et al. 2003) and, thus not necessarily a result of variation in sperm proteins (e.g. Hellberg and Vacquier 1999; Palumbi 1999; Swanson and Vacquier 1998). Rawson et al. (2003) conclude that the ability of some *Mytilus edulis* females to be fertilized by *M. trossulus* males provides a mechanistic explanation of the hybrids found in sympatric populations within the Gulf of Maine.

The presence of variable compatibility in some *M. edulis* females, coupled with an otherwise high level of gamete incompatibility, hybrid zone bimodality, a temporal overlap in spawning, and the presence of hybrids in natural populations, collectively suggest these females may be an indication of a process of reinforcement. In other words, these females are a remnant of the strengthening of prezygotic isolating barriers following secondary contact. In Rawson et al. (2003) only mussels from sympatric populations within the Gulf of Maine were used in cross-fertilization experiments, although the range of *Mytilus edulis* extends from the Canadian Maritimes to as far south as Cape Hatteras, NC. Thus, what is not known, to date, is the frequency of the atypically compatible females throughout the geographic range of *Mytilus edulis,* or the level of reproductive isolation between *M. edulis* and *M. trossulus* in allopatry.

A test of reinforcement would require that *in vitro* fertilizations were performed with *Mytilus edulis* and *M. trossulus* from both allopatric and sympatric populations across the species range. Evidence consistent with the outcome of reinforcement would include a signal of RCD, here as an increase in the atypically compatible phenotype in *M. edulis* females, or a general increase in heterospecific compatibility, in females found outside the hybrid zone. Moreover, selection against hybrids could be inferred from levels of introgression within sympatric populations, the structure of the hybrid zone, and through evidence of reduced hybrid viability from back-crosses of hybrid individuals (Jiggins and Mallet 2000; Marshall et al. 2002). Finally,

under a theory of reinforcement hybrid fitness should be reduced relative to that of the parentals. Hybrid fitness has not been directly address in any fertilization experiments to date. Reproductive isolating mechanisms between taxa can be developed and perfected by hybridization if the hybridization is relatively rare, with assortative mating reducing gamete wastage from the production of inviable hybrids.

Blue mussel hybridization provides an excellent model system with which to address the evolutionary formation, maintenance, and perfection of reproductive isolation through gametic incompatibility. Although hybridization between species in the *Mytilus edulis* complex is frequent, in all cases the parental taxa maintain their integrity, even in the most geographically expansive hybrid zones. The *Mytilus edulis – M. trossulus* hybrid zone within the Gulf of Maine is advantageous for a study addressing reinforcement because it provides the opportunity to examine features inherent to the process of reinforcement, in this case the prezygotic isolating trait of gamete incompatibility.

For Gulf of Maine populations, limited introgression suggests a selection against hybrids (Rawson 2001), and further investigation of gametic incompatibility between northwestern Atlantic populations of *Mytilus edulis* and *M. trossulus* may provide support of reinforcement by isolation. This study was designed to address RCD using reproductively isolating traits in a free-spawning marine invertebrate species-pair, where hybrids are found in natural populations. A simple comparison was made evaluating the frequency of atypically compatible females, and levels of heterospecific gamete compatibility, in an allopatric population compared to a sympatric, hybridizing, population.

MATERIALS AND METHODS

Sample and collection

Mussels used in fertilization assays were collected from two locations in the Gulf of Maine over a two week period from June 6-19 in 2003 and 2004, when gonad condition indicated both males and females were prepared to spawn. Collections were made in the East Bay region of Cobscook Bay (CB) in eastern Maine (latitude 44°52′50″N; longitude 67°07′13″W, two sites in 2003, one site in 2004) where *Mytilus edulis* and *M. trossulus* co-occur and hybrids are documented at 12-13% (Rawson et al. 2001), and they were also collected from coastal Kittery (K) in southern Maine (latitude 43°04′04″N; longitude 70°41′20″W, one site in 2003, one site in 2004), an allopatric population of *Mytilus edulis* (Figure 1). The Cobscook Bay sites were characterized by tidal mud flats, where the mussels formed extensive reefs, while both Kittery sites were rocky *Ascophyllum* dominated outcroppings on sand beaches. In 2003, a third site in the John's Bay region (RH) in the mid-Gulf of Maine (latitude 43°51′15″N; longitude 69°31′58″W) was sampled, however one half the individuals spawned from this site demonstrated contamination in fertilization controls (see *Fertilization; data collection and analysis*), and the remaining individuals from this site were dropped from subsequent analysis.

At each site approximately 200 adult mussels (50-80 mm shell length) were randomly collected along two 10m transect lines placed parallel to the shoreline at lowest tide. Following collection, mussels were transported back to the Darling Marine Center, Walpole, ME (2003) or the University of New England Marine Science Center,

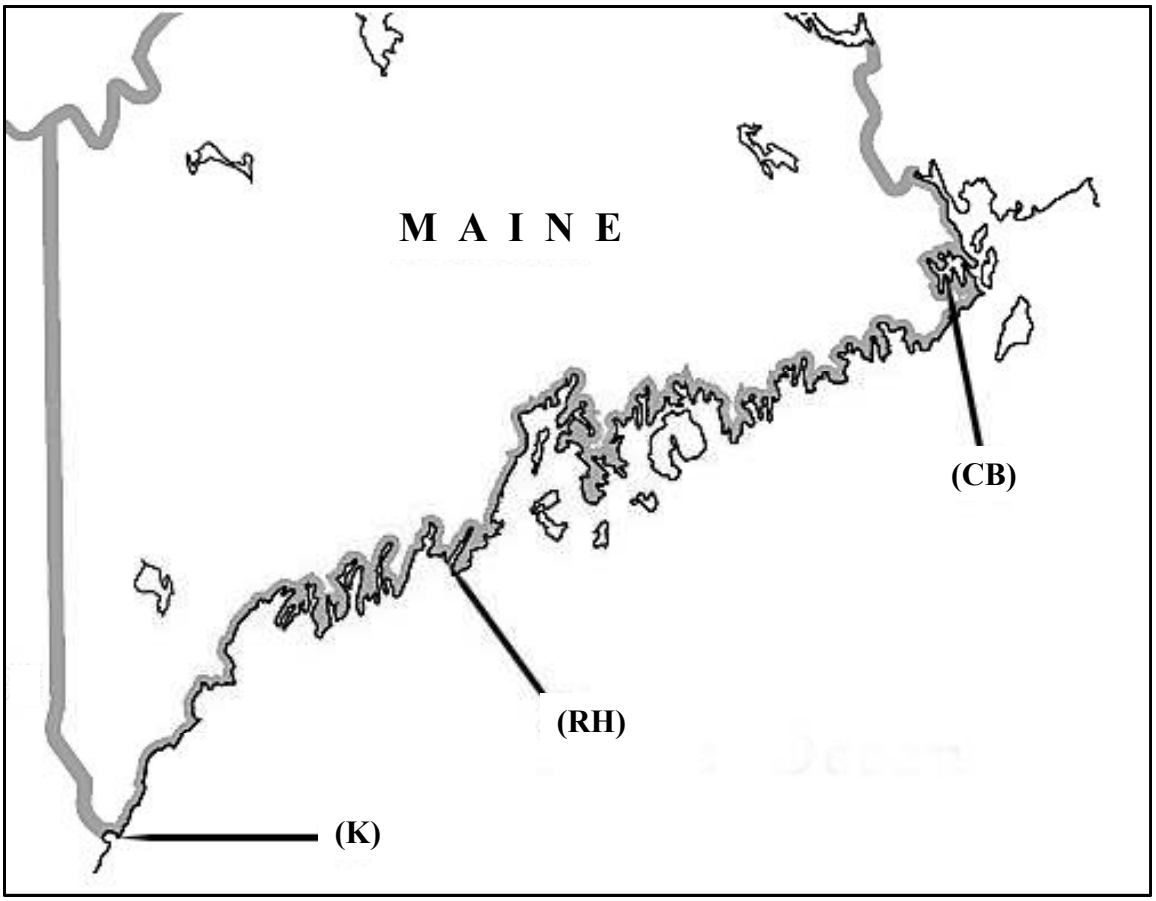


Figure 1. Collection sites for *Mytilus edulis*, *M. trossulus* and hybrid individuals used in *in vitro* fertilization experiments. Two sites within the Cobscook Bay region (**CB**) and two sites at Kittery (**K**), Maine were sampled in 2003 and 2004. One site was sampled in the John's Bay region (**RH**) in 2003.

Biddeford, ME (2004), where they were separated by site and maintained in a static seawater system at 9°C for identification and subsequent spawning. Mussels were fed daily with an *Isochrysis galbana, Pavlova lutheri,* and *Nannochloropsis oculata* mixture. (Algal paste; Innovative Aquaculture, Lasqueti Island, British Columbia, Canada).

Genetic identification

The tissue used in genetic assays was obtained by inserting a wooden peg between the mussel's valves and by clipping a small piece of the mantle frill. Following tissue sampling, individuals were marked with bee-tags for later identification and spawning. Genomic DNA was extracted using a modification of the "Rapid Isolation of Mammalian DNA" protocol (Sambrook and Russell 2000). Individuals were initially identified to species using between one and three nuclear DNA PCR-based markers that are diagnostic for *Mytilus edulis* and *M. trossulus*: Glu 5' (Rawson et al. 1996; modifications in Appendix A), ITS (Heath et al. 1995) and Mal I (Rawson et al. 2001). In order to confirm that the Kittery population sampled was an allopatric site for *M. edulis,* a number of individuals (n = 28) were identified using between one and three of the above nuclear DNA PCR-based markers. The identity of individuals determined at one locus was later checked using all three nuclear loci. Hybrids were identified as either heterozygous at one or more loci, or having differing species-specific markers at two or more loci. The genetic identity of individuals used in *in vitro* fertilization experiments was later re-confirmed using the three nDNA PCR-based markers and an additional mitochondrial marker, 16S-F (Rawson and Hilbish 1995).

Spawning and crosses

In the 2003 experiments, mussels were spawned on two days, July 9 (d1) and July 11 (d2), and in 2004 spawning was staged on July 15 (d1-04). In both seasons the mussels were induced to spawn approximately one month following the date of collection. Gametes were obtained through one of two methods; for females, spawning was induced through temperature cycling and for the males used in the crosses, sperm was obtained through strip spawning (Rawson et al. 2003). In either case, in order to identify gender, individual mussels were submerged in separate plastic containers filled with aged seawater that were then alternately placed in a warm water bath (20-30°C) followed by an ice bath (0-4°C), each temperature for 30-40 min. Females that began to release eggs had their container moved to room temperature conditions while they continued to produce eggs; males that released sperm were removed immediately from their container, wrapped in a damp paper towel and placed on ice. In 2003 a small number of identified hybrids spawned and were used in crosses to "pure" males or females and to other hybrids (Appendix C). In 2004, interpopulation crosses (i.e. crosses between *M. edulis* from Cobscook Bay and Kittery populations) were performed for a small number of individuals (Appendix D). In both 2003 and 2004, *Mytilus trossulus* females that spawned were crossed with *M. trossulus* males used in heterospecific crosses in order to assess sperm quality of those males, as well as being used in crosses to hybrid individuals. The adductor muscle of all spawned individuals was taken for later confirmation of species identification.

Eggs obtained from each female were washed in aged seawater (ASW), allowed to settle, measured volumetrically, and resuspended at a 2% volume to volume in ASW in a 15 ml conical vial, then held on ice while sperm was prepared for the cross. Eggs were held between 5-28 hours (mean 12 ± 6 hr) before being used in crosses. Linear regression analysis showed no

dependence of sperm concentration necessary to achieve 20% fertilization (as measured by $\log F_{20}$; *see Fertilization; data collection and analysis*) on the duration eggs were held, although, in general, within each site the ability of conspecific sperm to fertilize eggs increased with the duration eggs were held, while the ability of heterospecific sperm to fertilize eggs decreased (Figure 2). Sperm was obtained by opening the mussel and making a small incision in the mantle tissue to allow the sperm to flow into a 1.5 ml centrifuge tube, which was capped and held on ice until use. This so called "dry sperm" was typically held no longer than the average time necessary to set-up and execute fertilization assays.

Crosses were performed by pipetting 500 µl of the egg/ASW suspension into six scintillation vials, each containing 4 ml of ASW. Sperm was serially diluted in separate dram vials by pipetting 100 µl dry sperm into 900 µl ASW, mixing, and adding 100 µl of this dilute sperm to the next vial. This same serial dilution was repeated across 5 orders of magnitude for each cross. For each serial dilution, 100 µl of each sperm suspension was added to scintillation vials containing eggs, and the vials were gently swirled and loosely capped. Two 50 µl subsamples of the third serial dilution were taken from each cross and fixed (1:1) in 2% gluteraldehyde, for subsequent direct counts and sperm concentration calculations.

Embryos were allowed to develop to 4-16 cell divisions (7.15 ± 1.10 hrs, 2003 and 12.30 ± 0.38 hrs, 2004). Development was stopped by the addition of 1 ml, 37% formaldehyde to each vial. "Egg-only" controls were created for the majority of females used (500 µl eggs/seawater into 4 ml aged SW). These control vials were fixed with 1 ml, 37% formaldehyde at the same time as the crosses involving each female were stopped. Across the two seasons a total of 123 crosses were performed using *M. edulis* females, 6 from CB, 4 from RH and 14 from K sites,

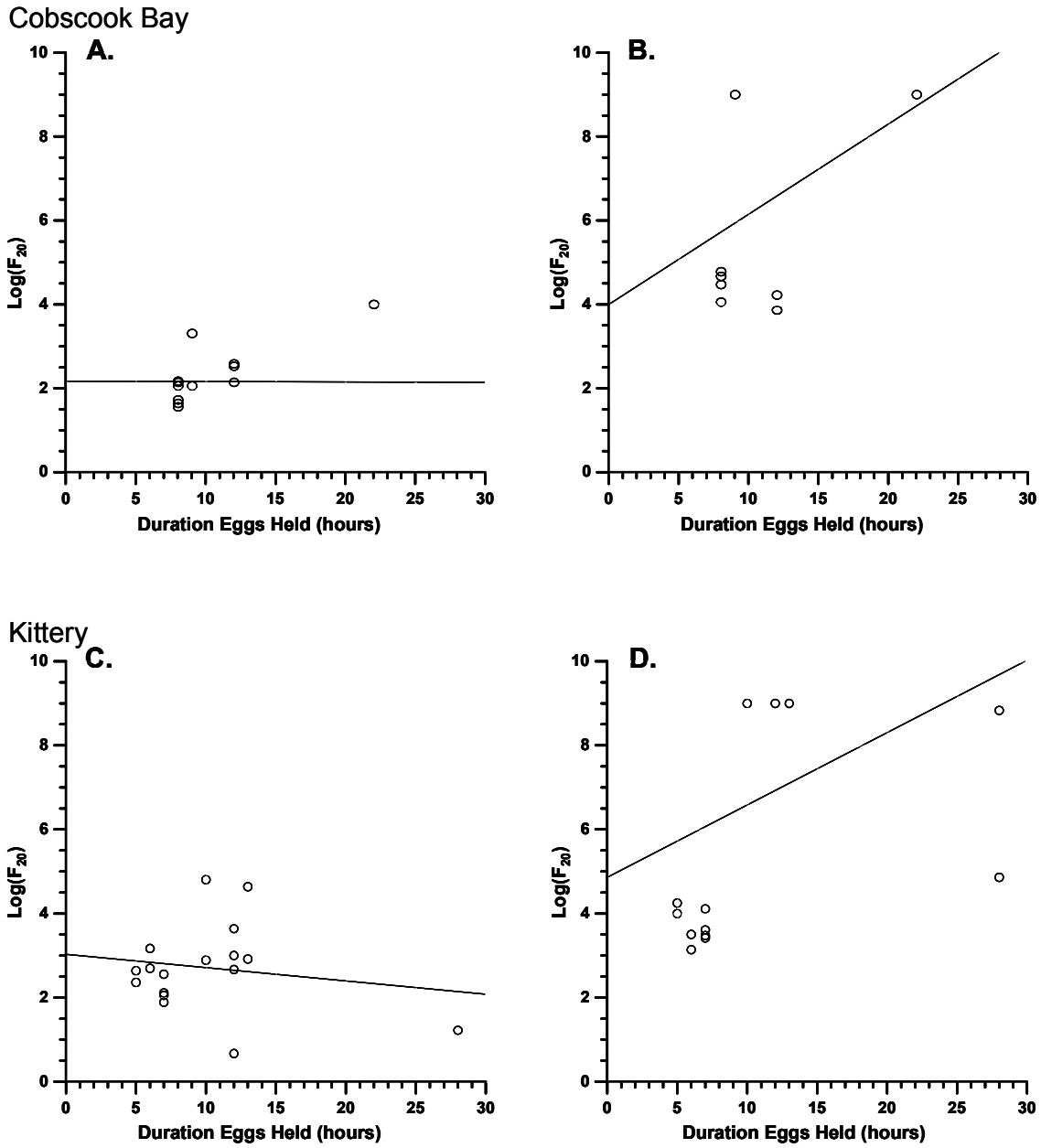


Figure 2. Linear regression analysis of log(F_{20}) to duration of time eggs were held prior to addition of sperm during *in vitro* fertilization assays. Conspecific (**A** and **C**) and heterospecific (**C** and **D**) regressions showed no strong dependence of log(F_{20}) on duration eggs were held (**A**, R^2=0.00, P=0.97; **B**, R^2=0.27, P=0.08; **C**, R^2=0.03, P=0.52; **D**, R^2=0.17, P=0.07).

respectively (total of 190 crosses performed; n = 50 hybrid crosses, n = 17 *M. trossulus* female crosses). Each female was crossed to only one male at a time and the sperm from some males (fresh serial dilutions) was used in more than one cross (Table 1). All *Mytilus trossulus* males used in heterospecific crosses were collected from populations sympatric with *M. edulis* at the Cobscook Bay sites.

Fertilization; data collection and analysis

Fertilization levels were determined by removing a 200 µl sub-sample of egg/ASW suspension and examining it on a microscope slide using a compound microscope (Olympus CH-2, x400 magnification). Approximately the first 200 eggs encountered were scored as either cleaving (4-16 cell divisions) or not cleaving, as a proxy for fertilization because no fertilization membrane is visible in *Mytilus.* Females that showed contamination (fertilized embryos) in their control vials, or that failed to show ≥ 70% fertilization at the highest sperm concentration in conspecific crosses, were dropped from subsequent analysis (2 RH females, 2 K females).

Sperm concentrations of male seminal fluid ("dry" sperm) were calculated from direct counts made using a Neubauer hemacytometer. In 2003, dry sperm concentrations were back calculated from a 10 µl sample of seawater taken from the fertilization scintillation vial containing the most concentrated sperm addition, and the concentrations for the series in that fertilization trial were calculated as 10 fold dilutions of that estimated dry sperm concentration. In 2004, the third sperm dilution in the dram vial was fixed, and subsamples of this suspension were directly counted and back calculations to dry sperm were made (Appendix B).

Table 1. Summary of *Mytilus edulis* con- and heterospecific fertilization data for crosses preformed on July 9, 2003 (d1), July 12, 2003 (d2) and July 17, 2004 (d1-04). Cobscook Bay *M. edulis* females are in the upper panel, Kittery *M. edulis* females are in the lower panel. The upper value: adjusted R^2 value associated with the linear regression of logit(proportion of eggs fertilized) on log(sperm concentration) for each cross preformed. Lower value: *F*-ratio testing of significance of the regression coefficient (**P*<0.05; ***P*<0.01; ****P*<0.001; NS not significant). B = crosses where no eggs were fertilized in any of the serial sperm dilutions.

Female	Male								
	M. edulis			*M. trossulus*					
Cobscook Bay *M. edulis*	Y55	O25		Y21	Y96	Y27	Y42	O35	Y30
Y71(d1)	0.962	0.856		0.639	0.613	0.286			
	127.69***	30.78**		9.86*	8.94*	3.00^{NS}			
G42(d1)	0.882	0.876		0.070	B	B			
	38.4**	36.41**		1.38^{NS}	B	B			
Y23(d2)		0.888					0.954	0.720	0.772
		40.93**					106.75***	13.91*	17.54*
	B53	B82	B77	W78	W73				
B45(d1-04)	0.873	0.653	0.790	0.957	0.948				
	35.27**	10.42*	19.77*	141.49***	92.30***				
B20(d1-04)	0.823	0.488	0.829	0.938	0.968				
	19.54*	5.76^{NS}	25.39**	77.29***	151.79***				
W79(d1-04)	0.653	0.283	0.796	0.956	0.868				
	10.41*	2.98^{NS}	20.52*	108.94***	34.02**				

	M. edulis						*M. trossulus*		
Kittery *M. edulis*	WB edm8	WB edm9	WB edm10	WB edm11	WB edm13	WB edm14	Y21	Y96	Y27
WB edf1(d1)			0.764				0.731	0.164	0.054
			17.19*				14.59*	0.78^{NS}	0.23^{NS}
WB edf2(d1)		0.982		0.905			0.029	0.754	0.260
		224.51***		48.81**			1.15^{NS}	16.39*	2.76^{NS}
WB edf3(d1)				0.827		0.790	0.030	0.198	0.051
				24.96**		19.82*	1.16^{NS}	2.23^{NS}	0.22^{NS}
WB edf5(d1)		0.783				0.835	0.286	B	B
		19.05*				26.29**	3.00^{NS}	B	B

	M. edulis						*M. trossulus*		
Kittery	WB edm8	WB edm9	WB edm10	WB edm11	WB edm13	WB edm14	Y21	Y96	Y27
WB edf6(d1)	0.751				0.930		0.566	0.484	0.563
	16.10*				52.71**		7.52*	5.69^{NS}	7.46*
	WB edm16	WB edm17	WB edm19	WB edm20			Y42	O35	Y30
WB edf9 (d2)	0.928	0.911					0.593		
	65.67**	52.28**					8.31*		
WB edf10(d2)				0.812			0.949	0.986	
				22.64*			93.53**	358.79***	
WB edf13(d2)			0.665				0.741		0.893
			10.92*				15.33*		43.03**
	K edm2	K edm3					W78	W73	
M. edulis									
K edf1(d1-04)	0.956	0.740					0.940	0.928	
	87.97**	15.27*					48.20**	67.76**	
K edf2(d1-04)	0.979	0.784					0.899	0.847	
	184.97***	19.20*					45.48**	28.67**	
Kedf3(d1-04)	0.958	0.901					0.950	0.968	
	114.22***	46.65**					95.04***	153.94***	
Kedf4(d1-04)	0.944	0.831					0.798	0.873	
	84.90***	25.67**					20.73*	35.53**	

Fertilization curves were generated for each female by plotting the proportion of eggs fertilized (Y-axis) against sperm concentration (X-axis). In order to further examine the relationship between eggs fertilized and sperm concentration, a logit transformation was used (after McCartney and Lessios 2002; Rawson et al. 2003). Sperm concentrations for each dilution were log transformed and % fertilized egg (P) counts were logit transformed [logit (P) = ln (P/1 - P)]. Logit (P) values were then used as the dependant variable, and log sperm concentration as the independent variable, in linear regression for each cross. A factor of 0.01 was added to every value of P to allow for logit-transformation of crosses in which zero eggs were fertilized.

The degree of gametic compatibility in a cross type was quantified by estimating the sperm concentration needed to achieve 20% fertilization (F_{20}) from the logit regressions. F_{20} was used rather than F_{50} (McCartney and Lessios 2002; Levitan 2002) because many heterospecific crosses failed to achieve >20% fertilization at the highest sperm concentration and using F_{50} would have required extrapolation beyond the maximum P value found in many heterospecific crosses. In evaluating heterospecific crosses where the calculated F_{20} value exceeded that of the highest sperm concentration used in the cross (defined as "blocked crosses" *see Results-Fertilization curves)*, or in the case when calculation of F_{20} resulted in a biologically unrealistic sperm concentration, the F_{20} value was set to equal the mean concentration of undiluted dry sperm used in that series of crosses. While this procedure is artificial in that an F_{20} of blocked crosses cannot truly be estimated, it did allow these crosses to be included in analysis in which other crosses showed only partial compatibility.

In order to achieve a near equal number of females crossed (and number of crosses) within the Cobscook Bay sites compared to the Kittery sites, conspecific and heterospecific crosses involving *M. edulis* females from a 2001 experiment (Rawson et al. 2003) were added to

the 2003-2004 data sets. In both 2001, and later 2003-04 experiments, gametes were obtained from individuals collected at the same site (i.e. one site within Cobscook Bay), individuals were housed, spawned, and crosses were performed in the same manner. However in 2001 experiments, each cross was replicated so only one of each of the replicated crosses was haphazardly chosen and added to the 2003-04 data set.

Given that the F_{20} values of heterospecific crosses failed to achieve normality following log transformation, a quantitative comparison of F_{20} values was conducted using non-parametric statistics analogous to a one-way ANOVA. A plot of the distribution of F_{20} values indicated that both statistics of location and dispersion were of interest when analyzing levels of gamete compatibility and differences between sites. A Kruskal-Wallis test, sensitive to location (Sokal and Rohlf 1995), was used to test for effect of year and female on F_{20} values within each of the two sites. The Mann-Whitney U two-sample test was used to compare the effect of cross-type on F_{20} values within each of the two sites, and an effect of site on F_{20} was examined between sites. The distribution of both conspecific and heterospecific F_{20} values were compared between sites using a Kolmogorov-Smirnov two-sample test, which is sensitive to differences in dispersion (Sokal and Rohlf 1995). A comparison of frequency of heterospecific blocked crosses was made between site of collection using a *G*-test of independence and the *G* values were adjusted using Williams's correction (Sokal and Rohlf 1995). The linear regressions, Kruskal-Wallis test, Mann-Whitney U, and Kolmogorov-Smirnov two-sample test were preformed using SYSTAT 11.0 (SPSS, Chicago).

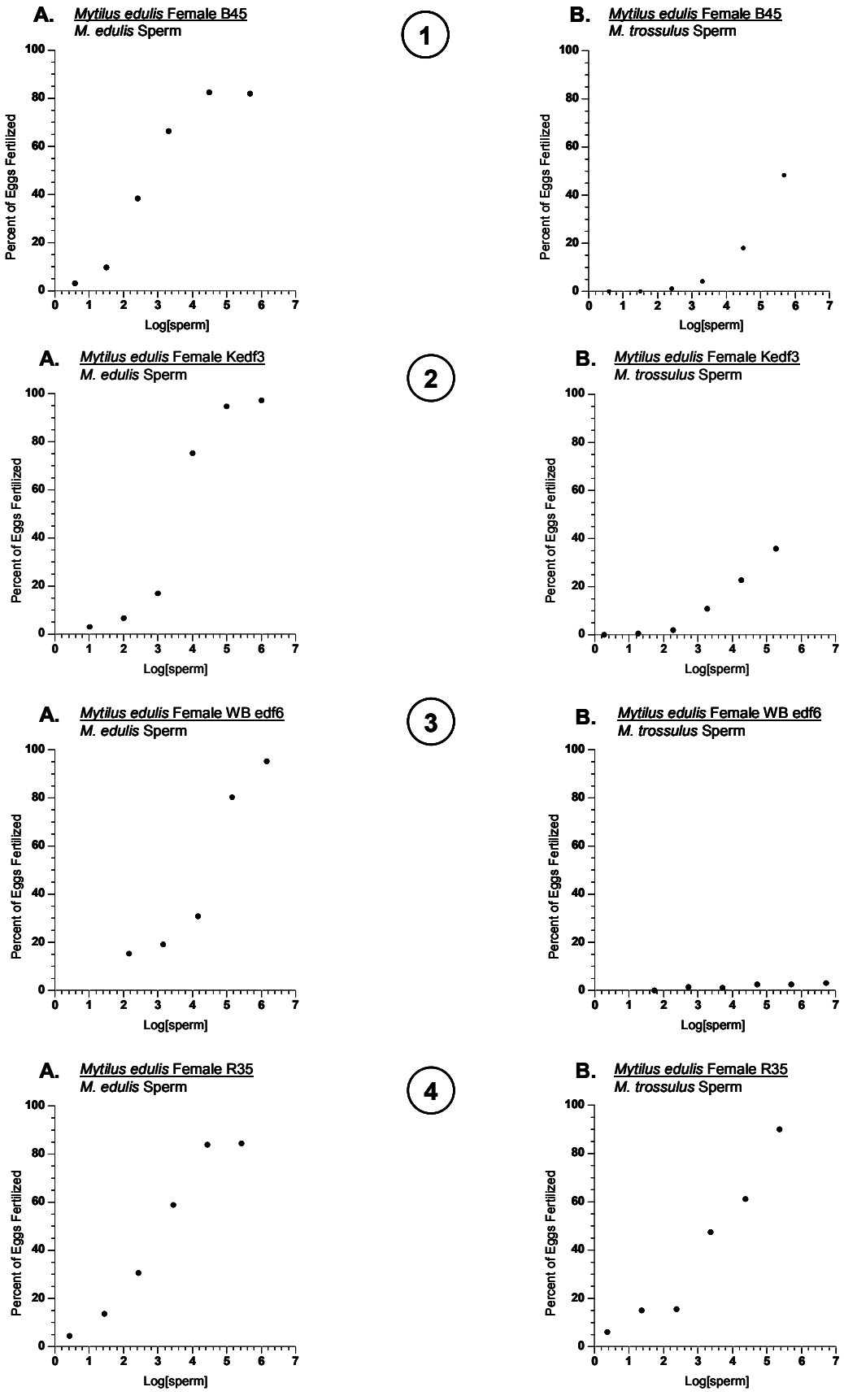
RESULTS

Fertilization curves

Conspecific fertilization curves generated for *Mytilus edulis* mussels at the two sites were similar to one another and consistent with past fertilization experiments involving *M. edulis* sperm and eggs from within the Cobscook Bay region (Rawson et al. 2003, Fig. 1). At both the Cobscook Bay and Kittery sites, the proportion of eggs fertilized rapidly increased with sperm concentrations between 10^3-10^4 ml^{-1} (Figure 3), and in general, conspecific crosses showed ≥ 80% fertilization at sperm concentrations of 10^6 ml^{-1} or greater. In contrast, in most cases, heterospecific crosses failed to generate fertilization levels greater than 20% at those same sperm concentrations. Equally, there appeared a large proportion of "blocked crosses" – defined as heterospecific crosses that failed to achieve greater than 10% fertilization at the highest sperm concentration (10^6-10^7 ml^{-1}) (Figure 3).

The linear regression of logit-transformed fertilization estimates showed a strong dependence of fertilization on sperm concentration. At the Cobscook Bay site, the majority of conspecific crosses had an R^2 value exceeding 0.8 and the linear regression between logit-transformed fertilization and log transformed sperm concentration was statistically significant in 12 of the 14 crosses preformed (Table 1). For the two conspecific crosses that were not significant linear regressions, it is likely that this outcome was the result of depression in the fertilization curves at high sperm concentrations, rather than an overall poor relationship between proportion of eggs

Figure 3. Characteristic results from *in vitro* fertilizations involving *Mytilus edulis* females performed across a series of sperm dilutions. (A): percent of eggs fertilized plotted as a function of log-sperm concentration (sperm ml^{-1}) for conspecific crosses from CB sites (1 and 4) and K sites (2 and 3). (B): each female's fertilization curve when crossed with heterospecific sperm. 3B: representative of a "blocked cross." 4B: heterospecific fertilization curve for a female displaying the atypically compatible phenotype.


A.
Mytilus edulis Female B45
M. edulis Sperm
1
B.
Mytilus edulis Female B45
M. trossulus Sperm
A.
Mytilus edulis Female Kedf3
M. edulis Sperm
2
B.
Mytilus edulis Female Kedf3
M. trossulus Sperm
A.
Mytilus edulis Female WB edf6
M. edulis Sperm
3
B.
Mytilus edulis Female WB edf6
M. trossulus Sperm
A.
Mytilus edulis Female R35
M. edulis Sperm
4
B.
Mytilus edulis Female R35
M. trossulus Sperm
Percent of Eggs Fertilized
Log[sperm]

fertilized and sperm concentrations (Figure 4). These females did show significant regressions when crossed with two other conspecific males (Figure 4) and if the data point at the highest sperm concentration was removed, each regression became significant. In both cases the nonsignficant regression did not substantially alter the estimated F_{20} value for each cross (Figure 4). For the Kittery site *Mytilus edulis* females, all regressions of logit(proportion of eggs fertilized) on log(sperm concentration) were significant, with a majority of adjusted R^2 values exceeding 0.80. Heterospecific crosses between *M. edulis* females and *M. trossulus* males generated significant regressions in the majority of crosses performed at each site with R^2 values between 0.56 – 0.99 (Table 1). Nonsignificant regressions are not uncommon for fertilization curves generated from heterospecific crosses (McCartney and Lessios 2002), although some blocked crosses did show a dependence of fertilization on sperm concentration (Table 1; WB edf1 n=1, WB edf2 n=1, WB edf6 n=2).

F_{20} and site differences

Analysis of the F_{20} values calculated from the logit regressions indicated that *Mytilus edulis* females at both sites have strong blocks to heterospecific fertilization. Crosses involving Kittery site *Mytilus edulis* females showed the strongest blocks, requiring as much as a 76 thousand-fold increase (as estimated by comparison of mean F_{20} values) in *M. trossulus* sperm in order to achieve fertilization similar to that of crosses with *M. edulis*. Cobscook Bay *M. edulis* females required considerably less *M. trossulus* sperm for heterospecific fertilization levels similar to conspecific in comparison to those calculated for the Kittery females. For the Cobscook Bay site, F_{20} analysis

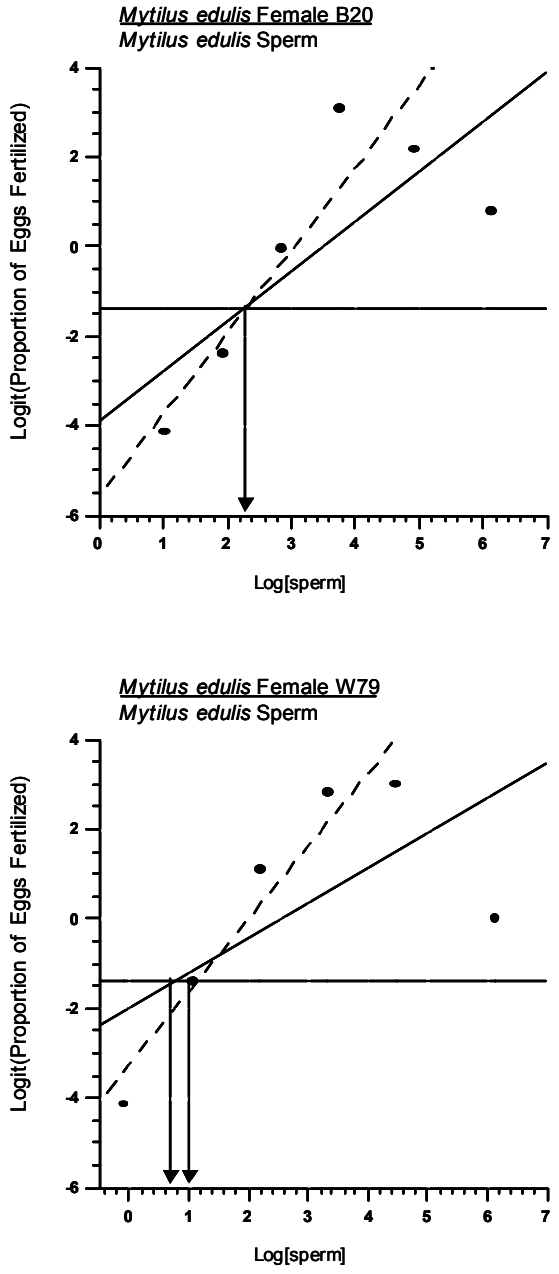


Figure 4. Linear regressions of logit(proportion of eggs fertilized) on log(sperm concentration) for two Cobscook Bay *Mytilus edulis* females that showed depression in their fertilization curves at high sperm concentrations. Solid lines: best fit line from linear regression before the data point at the highest sperm concentration is removed. Dashed lines: best fit line from linear regression after the data point is removed. Line parallel to the x-axis at –1.37 = logit(0.20), or 20% fertilization

nonetheless showed an estimated 3400-fold increase is required for heterospecific fertilization (Table 2). The Mann-Whitney U (i.e. Kruskall-Wallis with two samples) and Kolmogorov-Smirnov tests indicated there was no significant difference in conspecific F_{20} values between sites (U=159.00, P=0.080; D=0.348, P=0.137). In contrast, heterospecific F_{20} values were significantly different from conspecific values within sites (CB: U=46.00, P<0.001, D=0.808, P<0.001; K: U=42.00, P<0.001, D=0.726, P<0.001). Also the distribution of heterospecific F_{20} values differed significantly between sites (D=0.409, P=0.018) Equally, there appeared a significant effect of year on heterospecific F_{20} values within each site (Table 3).

It is likely that the highly significant difference between conspecific and heterospecific $F_{20,}$ the significant effect of year, as well as the high estimates of *M. trossulus* sperm concentration necessary to achieve fertilization similar to conspecific crosses is due to the presence of blocked crosses in females from both sites sampled. From a total of 26 heterospecific crosses analyzed using Cobscook Bay *Mytilus edulis* females, 7 (1 cross from 2001 and 6 crosses from 2003) showed less than 10% fertilization at the highest sperm concentration resulting in an F_{20} assignment of the log(mean dry sperm concentration). In contrast, over twice as many heterospecific crosses were blocked in *M. edulis* females from the Kittery site, 54% of the 28 heterospecific crosses analyzed (Figure 5) – all found in 2003 crosses (Table 3). This difference in number of heterospecific blocked crosses compared between sites was significant (G – test, $G_{adj.}$ = 3.92, P<0.05). In addition, this difference is mirrored in the F_{20} ratio compared between sites, with Kittery heterospecific crosses requiring nearly 41% more sperm than Cobscook Bay heterospecific crosses to achieve 20% fertilization

Table 2. Summary and comparison of *Mytilus* spp. F_{20} values estimated from logit regressions for the two crossing types. Cross types are indicated with the female used in the cross listed first, followed by the male species used in the cross (E = *M. edulis*, T= *M. trossulus*). Mean F_{20} values and 95% confidence limits (CL) are reported as back-transformed from their logs. The F_{20} ratio describes the increase in sperm concentration necessary to achieve 20% fertilization in heterospecific crosses over that necessary in a conspecific cross (NA = not applicable). Lower table represents heterospecific crosses tested after blocked crosses were removed.

Cross Type	Site	*n*	F_{20}	Upper CL	Lower CL	F_{20} ratio
E x E	CB	22	0.82×10^{2}	3.47×10^{2}	0.19×10^{2}	NA
E x T	CB	26	2.80×10^{5}	3.27×10^{6}	2.39×10^{4}	3.40×10^{3}
E x E	K	21	1.49×10^{2}	3.18×10^{2}	0.70×10^{1}	NA
E x T	K	28	1.14×10^{7}	9.98×10^{7}	1.29×10^{6}	7.60×10^{4}
CB_{ExE} K_{ExE}						
CB_{ExT} K_{ExT}						
E x T_{NB}	CB	19	1.29×10^{4}	8.04×10^{4}	2.01×10^{3}	157
E x T_{NB}	K	13	5.6×10^{4}	5.18×10^{5}	6.08×10^{3}	375

Table 3. Results of the Kruskal-Wallis one-way analysis of variance testing the effects of an individual female used in the cross, and year the cross was preformed on log(F_{20}) values. Conspecific crosses pooled between sites appear as "pooled" under the site heading. Females used in each site; CB *n*=12, K *n*=12. Years crosses were performed; CB *n*=3, K *n*=2. The Kruskal-Wallis test statistic (*H*) is preceded by number of cases in parentheses. Cross type as described in Table 2 legend.

Site	Effect	Cross type	***H***	*P*
CB	Female	ExE	NA	
CB	Female	ExT	(26) 21.83	0.026
CB	Year	ExE	NA	
CB	Year	ExT	(26) 5.00	0.082
K	Female	ExE	NA	
K	Female	ExT	(28) 25.92	0.007
K	Year	ExE	NA	
K	Year	ExT	(28) 160.00	<0.001
Pooled	Female	ExE	(43) 28.60	0.235
Pooled	Year	ExE	(43) 1.48	0.477

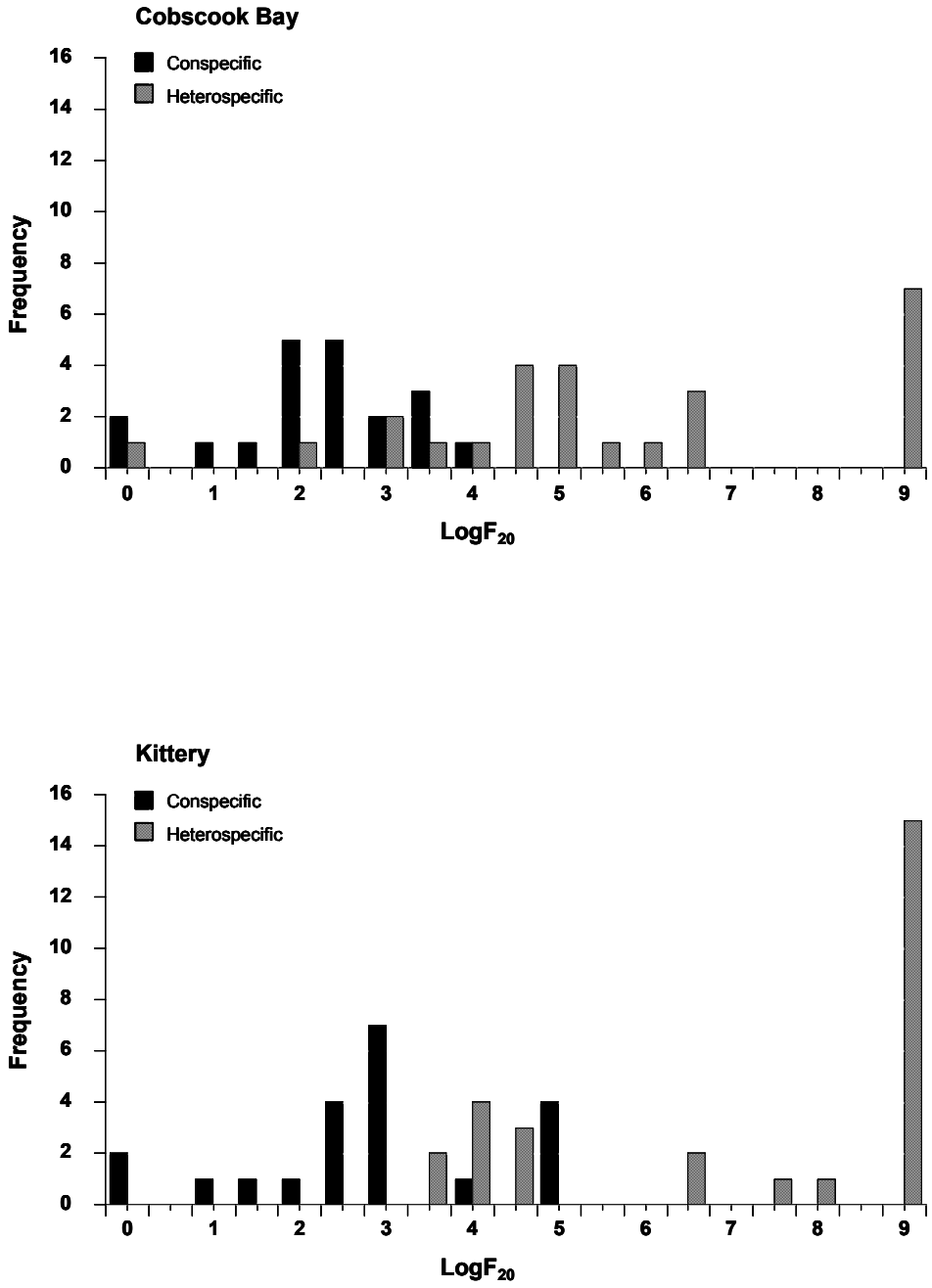


Figure 5. The frequency of occurrence of log(F_{20}) values for each cross type plotted within site. Crosses with a log(F_{20}) value of 9.0 are blocked crosses. Cobscook Bay site: conspecific cross (E_{Female} x E_{Male}) n = 22, heterospecific cross (E_{Female} x T_{Male}) n = 26. Kittery site: conspecific cross (E_{Female} x E_{Male}) n = 21, heterospecific cross (E_{Female} x T_{Male}) n = 28.

(Table 2). It is possible that the presence of blocked crosses in estimates of F_{20} influences this difference. The removal of blocked cross F_{20} estimates from calculation of mean heterospecific F_{20} yields a greater similarity between sites, although the heterospecific F_{20} ratio in Cobscook Bay crosses is much less than that found in the past (Rawson et al. 2001, *Table 3*) (Table 2). Removal of blocked crosses from analysis of estimated F_{20} values did not, however, eliminate the significant difference between conspecific and heterospecific cross F_{20} within site (Table 2).

The degree of heterospecific gamete compatibility was greater among Cobscook Bay *Mytilus edulis* females compared to those of Kittery site females, with a majority of estimated F_{20} non-blocked heterospecific falling between $10^3 – 10^6$ sperm ml^{-1}. That same estimated F_{20} for Kittery site females fell across a relatively narrower and higher range of values, between $10^4 – 10^8$ sperm ml^{-1} (Figure 5). Moreover, only in the Cobscook Bay site crosses was there an overlap of F_{20} values between conspecific and heterospecific crosses at a level that would suggest partial compatibility of *Mytilus edulis* eggs with *M. trossulus* sperm (Figure 5).

Patterns in the atypically compatible females

Previous identification of partial gametic compatibility in *Mytilus edulis* females within the Gulf of Maine has come from comparison of each female's mean heterospecific F_{20} to the mean F_{20} for conspecific crosses (Rawson et al. 2003). For the present data set, within each site there was a significant effect of female on heterospecific F_{20} (Table 3). Again, this could be driven by the presence of blocked crosses within the data set, with the majority of blocked heterospecific crosses occurring in the 2003 fertilization experiments. However, a comparison of each female's mean $\log(F_{20})$ value to

the mean conspecific log(F_{20}) within site indicates that for the Cobscook Bay site there is also a great deal of variation among individual females in degree of heterospecific gametic compatibility, whereas the estimated log(F_{20}) values for females from the Kittery site showed a trend towards a bimodal distribution (either blocked or with heterospecific log(F_{20}) values between 4-6 (10^4-10^6 sperm ml^{-1})(Figure 5).

Within the Cobscook Bay site, there are only two females with mean heterospecific F_{20} that are statistically indistinguishable from mean conspecific F_{20} – R35 (2001) and Y23 (2003)(Figure 5), although a plot of log(F_{20}) frequency suggests that many more females have heterospecific log(F_{20}) similar to conspecific log(F_{20}). In contrast, at the Kittery site there are no *M. edulis* females that show overlap in their heterospecific log(F_{20}) to that of the mean conspecific log(F_{20}) for the site (Figure 5). Two females from 2004 experiments, K edf1 and K edf4, have values that appear similar, but on a log scale these females would require 1000-fold more sperm to achieve fertilization levels that overlap the mean conspecific log(F_{20}) (Figure 6).

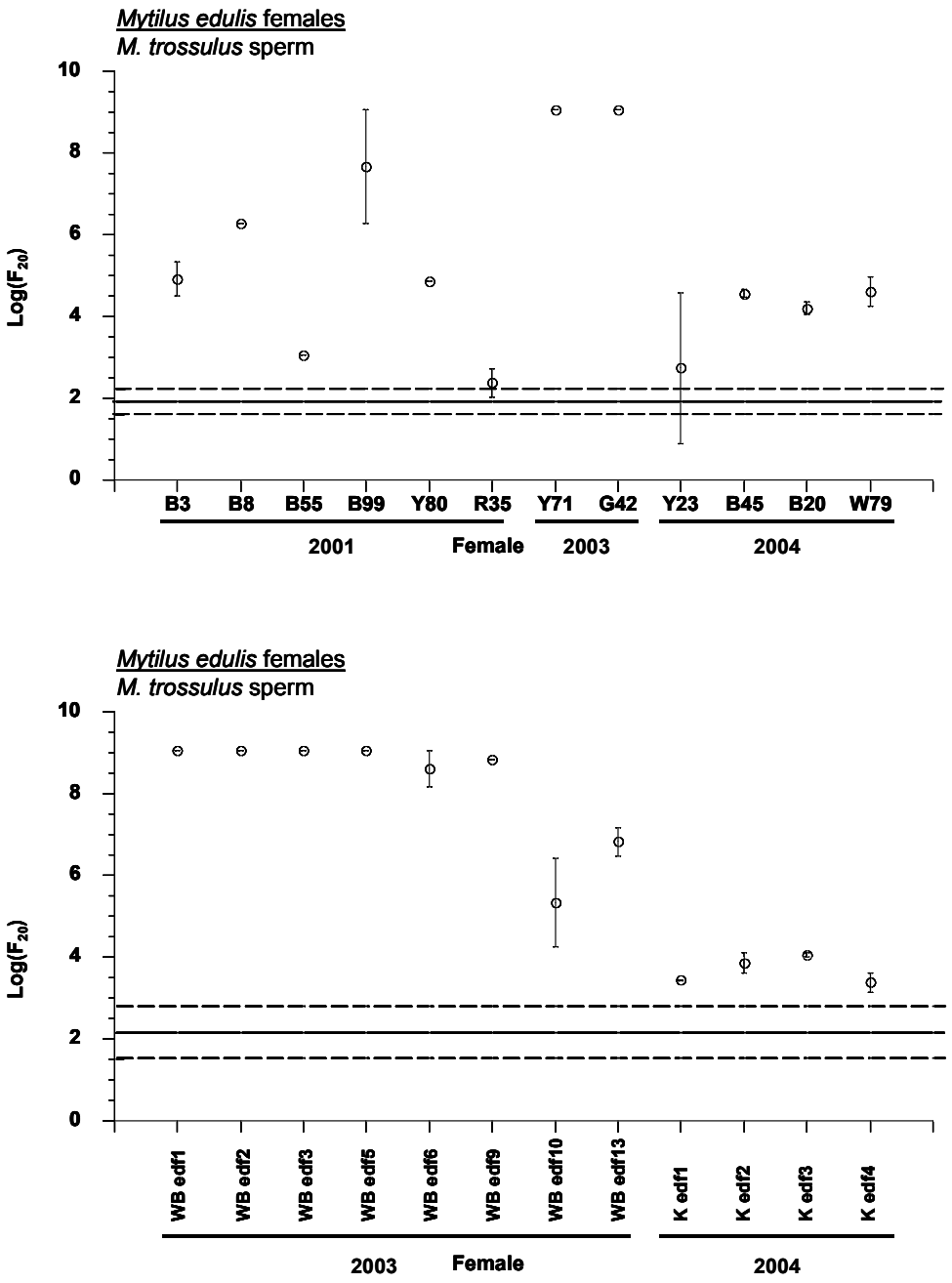


Figure 6. Mean log(F_{20}) (±SE) for *Mytilus edulis* females used in heterospecific crosses with *M. trossulus* males. Upper panel: CB sites females. Lower panel: K sites females. Solid line: mean conspecific log(F_{20}) for each site. Dashed lines: ±SE of that mean.

DISCUSSION

Fertilization curves and gamete compatibility

The high frequency of heterospecific blocked crosses between *Mytilus edulis* females and *M. trossulus* males indicates a high degree of gamete incompatibility between these two species, somewhat greater than previously estimated within the same area of sympatry (Rawson et al. 2003). A higher frequency of blocked heterospecific crosses was found when crossing *M. edulis* females from an allopatric population to *M. trossulus* males, indicating this gamete incompatibility is not restricted to sympatric populations. In the present study, excluding the 2001 data, a slightly higher number of *M. edulis* females from the Cobscook Bay sites were spawned then were used previously (7 compared to 5), and two of those females (Y71 and G42) (29%) showed this blocked trait whereas none were completely blocked in 2001. With all other experimental factors the same (i.e. population, spawning times and methods, *in vitro* fertilization assays), and with a good fit between logit(P) and log[sperm] in conspecific crosses in the present study, it is likely that increasing the sample size resulted in the identification of this trait. Poor gamete quality of the females displaying the trait is not indicated, as the conspecific F_{20} of these females was similar to "non-blocked" females . Alternatively, the significant effect of year on heterospecific F_{20} may be a factor, although with sample sites, collection times, culture and spawning conditions equal the mechanism driving this effect remains unclear.

Similar low levels of interspecific gamete compatibility has been documented in urchins (Strathmann 1981; Metz et al. 1994; Levitan 2002; McCartney and Lessios 2002), polychaetes (Pawlik 1988; Mardsen 1992; Pernet 1999), echinoids (McClary and Sewell 2003), and corals (Levitan et al. 2004). Non-functioning interactions between gamete recognition proteins are

thought to be responsible for heterospecific gamete failure in many closely related free-spawning invertebrate taxa (Metz et al. 1994; Vacquier 1998). For mussels within the *Mytilus edulis* complex, variation in selection pressures on the sperm acrosomal protein, M7 lysin (Riginos and McDonald 2003), as well as variation in compatibility loci in females, may be responsible for not only the failure of heterospecific sperm to fertilize eggs (Wu 1985), but also for variation in conspecific fertilization observed in some *M. edulis* females crossed. Equally, the depression observed in some conspecific fertilization curves at high sperm concentrations may be the result of polyspermy (Franke et al. 2002; Levitan 2004). Polyspermy in *Mytilus edulis* has been shown to result in failure or abnormal cleavage (Togo et al. 1995). Eggs displaying either of these characteristics would have been scored as "unfertilized" when encountered, however, fertilization curve depression, whatever the cause, did not significantly alter F_{20} estimates.

Evidence for RCD?

The theory of reinforcement (Dobzhansky 1937; Butlin 1989; Liou and Price 1994) suggests an expected outcome of RCD when prezygotic isolating traits are compared between sympatric populations and allopatric populations (Brown and Wilson 1956; *reviewed in* Howard 1993). As a result of natural selection against hybridization, individuals in sympatric populations would show greater prezygotic isolation than individuals in allopatric populations. This type of pattern has been observed in a variety of taxa (Howard 1993) and for free-spawning marine invertebrates is not unlikely. Evaluating patterns of DNA sequence divergence in gamete recognition proteins (bindin) in urchins, Geyer and Palumbi (2004) found evidence for RCD in a comparison of sympatric and allopatric populations of *Echinometra oblonga* in the Indo-west Pacific.

However, in the case of *Mytilus edulis* females within the Gulf of Maine, our analyses of F_{20} values from *in vitro* fertilization assays suggest a pattern opposite to that expected under a theory of reinforcement. Allopatric *M. edulis* females showed stronger heterospecific gamete incompatibility, with a mean heterospecific F_{20} forty-one times greater than that of the mean sympatric heterospecific F_{20}. This same pattern is reflected in blocked heterospecific crosses. Within the Kittery site, blocked crosses accounted for over half (54%) of the heterospecific crosses preformed, while within the Cobscook Bay site heterospecific blocked crosses occurred in a little over one-quarter (27%) of the total heterospecific crosses. Even following the removal of blocked crosses this pattern of greater heterospecific gamete incompatibility in the allopatric population is still present, with a mean heterospecific F_{20} value for the Kittery site crosses over four times that of the mean heterospecific F_{20} for the sympatric Cobscook Bay site crosses. Equally, the distribution of F_{20} values within each site shows that heterospecific crosses in allopatric populations are more frequently result in an F_{20} of 10^4 or greater, while that same cross type within sympatric populations have a broader distribution indicating weaker blocks to heterospecific fertilization for some *M. edulis* females.

Atypical compatibility in *Mytilus edulis*

Past research addressing gamete compatibility between *Mytilus edulis* and *M. trossulus* within the Gulf of Maine has defined *M. edulis* females with "atypical compatibility" in heterospecific crosses as having F_{20} values that do not differ significantly from the mean conspecific F_{20} (Rawson et al. 2003). If similarity to conspecific F_{20} is the measure, then this trait occurs at a much lower frequency within sympatric populations than previously estimated. From this study approximately 17% of *M. edulis* females (2 of 12) from a sympatric population

show extreme compatibility similar to that observed in Rawson et al. (2003). In contrast, this trait, as defined, is not observed in any of the geographically allopatric *M. edulis* females used in heterospecific crosses. However, the broad distribution of heterospecific F_{20} shows that compatibility is more better viewed as a quantitative character that varies in both sympatric and allopatric populations.

The sperm concentrations necessary to achieve 20% fertilization in the heterospecific crosses that were not "atypically" similar to conspecifics (i.e. 10^3-10^6 sperm ml^{-1}) may still be biologically meaningful. Levitan (2004) found from field spawning experiments that urchins experience high sperm concentrations (similar to laboratory conditions of 10^7 sperm ml^{-1}) when male densities increase. Given the aggregated nature of blue mussel beds it is not unlikely that females may experience high sperm concentrations during spawning. This could create the sperm concentrations necessary for high levels of heterospecific fertilization in eggs of these more compatible females, if indeed the timing of spawning overlaps, and in the absence of any evolved conspecific gamete preference. However, the relatively low frequency of F_1 hybrids coupled with the bimodal distribution of genotypes found in areas of coexistence suggest either, or both, small scale temporal differences in spawning and conspecific gamete preference may be acting to limit hybridization between *M. edulis* and *M. trossulus* (Jiggins and Mallet, 2000).

Conclusions and alternative hypotheses

The mechanisms promoting the patterns of interbreeding, and non-fusion of *Mytilus edulis* and *M. trossulus* within the Gulf of Maine populations remain unclear. Within this hybrid zone, the presence of atypically compatible females, bimodality of the hybrid zone and possible postzygotic selection against hybrids (see Toro et al. 2004 for discussion) suggest the process of

reinforcement may be acting to limit hybridization in sympatric populations. Yet, from this study there is an absence of a signal of reproductive character displacement. There are, however, several hypotheses that could explain not only a lack of enhanced isolation, but also other characteristics of the sympatric population.

To begin, it is possible that both the geographic pattern of heterospecific gamete incompatibility opposite to that expected under a theory of reinforcement, as well as the increased heterospecific gamete compatibility in sympatric *Mytilus edulis* females is the result of introgression within the hybrid zone populations. While a hybrid index constructed for this region shows limited introgression (Rawson et al. 2001), it is based on the use of 4 genetic markers, which were the same used in this study. Arguably, a hybrid index with as few as four markers could provide a coarse classification of individuals within a hybrid zone, but in order to obtain the resolution to construct a quantitative hybrid index capable of discriminating between advanced backcrosses and parental species, upwards of 20 markers may be required (Boecklen and Howard 1997). Theoretically, that number of markers could be obtained through the use of amplified fragment length polymorphisms (AFLP's) (Mueller and Wolfenbarger 1999) (Appendix F) and AFLP has been used to successfully differentiate species within many genetically diverse taxa (e.g. corals: Lopez et al. 1999; fishes: Campbell et al. 2003; plants: Kirk et al. 2004 .

A higher level of resolution obtained from screening a larger portion of the genome would allow the genetic and demographic consequences of introgression to be addressed as they are occurring during the evolution of novel hybrid genotypes. Additionally, evidence of unidirectional or extensive introgression may shed light on the mechanisms establishing and maintaining this hybrid zone. It is possible that reproductively isolating traits in *Mytilus edulis* in

the Gulf of Maine had evolved in allopatry prior to secondary contact with *M. trossulus* following a second trans-artic exchange (Riginos and Cunningham 2005). The atypically compatible trait displayed by some *M. edulis* females, then may be the product of ancient hybridization events, with introgression of gene regions that confer compatibility driving the pattern of increased heterospecific compatibility within the hybrid zone – but remaining undetected.

Under this scenario, initial introgression could have been facilitated not from *Mytilus edulis* females, but instead *M. trossulus* females which may not have evolved prezygotic isolating traits in allopatry, or initial hybridization was the result of a small number of founders interbreeding with locally abundant *M. edulis*. Despite the lack of evidence supporting the presence of the atypically compatible phenotype in *M. trossulus* females, the skewed nature of the hybrid zone index (i.e. higher frequency of *M. trossulus* alleles in hybrid genotypes) for the eastern Gulf of Maine (Rawson et al. 2001) suggests reproductive character displacement should be further evaluated for *M. trossulus*.

Alternatively, it is possible that populations of *Mytilus edulis* are not genetically isolated. Populations in Kittery, Maine and further south may experience a significant amount of gene flow from the sympatric population to the north, possibly from transport of pelagic larvae in the Eastern Maine Coastal Current (e.g. Townsend 1992). In this case, the absence of *Mytilus trossulus* adults from southern populations does not necessarily mean the *M. edulis* populations to the south of the hybrid zone are, or have been, evolving in allopatry. Although, Riginos et al. (2002) reject a hypothesis of Allopatric differentiation in the absence of hybridization for *M. edulis* has been rejected by Riginos et al. (2002) following genetic analysis of trans-Atlantic gene flow. Equally, the slight geographic differentiation among *Mytilus edulis* populations of North

America north and south of Cape Cod observed from allozyme analysis (Riginos and Cunningham 2005) was not seen in an initial study of cytochrome oxidase I sequences (Appendix E).

Gene flow, even at moderate levels could result in recombination of gene complexes, effectively countering any evolution of traits within geographically allopatric populations. However, under high to moderate gene flow, such as that likely for marine organisms with high dispersal potential, the expectation then would be no difference in prezygotic isolating traits between allopatric and sympatric populations. Although the two *M. edulis* populations used in this study may not be strictly allopatric, an outcome of no difference in prezygotic isolation was clearly not the case, with a significant difference found in heterospecific F_{20} values compared between sites. While a neutrally evolving mtDNA gene region provides a relatively good estimate of gene flow, genetic distances calculated from this region may not be indicative of reproductive (gamete) compatibility. Rather, sequence analysis of gene regions under selection (e.g. allozymes) or of the proteins responsible for gamete compatibility (e.g. lysin) may provide a better estimate of actual levels of interbreeding, as neutral regions may retain a signal of gene flow through ancient shared polymorphisms. In addition, the presence of bimodality within the hybrid zone may be a signal of prevention of recombination through assortative mating, countering disassociation of loci, even in the face of gene flow (Jiggins and Mallet, 2000).

Marshall et al. (2002) have suggested that the process of reinforcement is affected by both mating system patterns and gamete utilization. When multiple mating occurs within a hybrid zone, the cost of hybridization is reduced because males and females have the opportunity to be engaged in both conspecific matings and heterospecific matings, thus the strength of selection against hybridization may be weak. Multiple mating, coupled with conspecific gamete

preference is then predicted to reduce the likelihood of reinforcement (Marshall et al. 2002), and as such a signal of reproductive character displacement. Although the reproductive behavior of *Mytilus* spp in natural populations has yet to be documented, it is not inconceivable that eggs spawned come into contact with the sperm of multiple males, thus establishing a multiple mating system. Bierne et al. (2002) described assortative fertilization between *Mytilus edulis* and *M. galloprovincialis* from *in vitro* fertilization assays involving interspecific sperm competition. While the experiments preformed here were "no-choice", for *M. edulis* and *M. trossulus* within the Gulf of Maine, high levels of heterospecific gamete incompatibility coupled with hybrid zone bimodality suggest assortative mating (Jiggins and Mallet 2000). Equally, an overlap in gametogenesis and spawning (Maloy et al. 2003) supports opportunity for multiple mating, fulfilling both conditions for the prediction of absence of reinforcement made by Marshall et al. (2002).

Finally, if reinforcement is strictly defined as the strengthening of prezygotic isolating traits through the selection against hybridization (see Howard 1993), then evaluating the presence of reinforcement based on a pattern of RCD may result in a misleading conclusion. Lemmon et al. (2004) model a variety of circumstances where the process of reinforcement occurs in the absence of a signal of RCD, emphasizing that, in its strictest sense, the process of reinforcement strengthens prezygotic isolation relative to the level of prezygotic isolation when selection against hybrids does not occur. In order to determine if reinforcement has occurred in natural populations, Lemmon et al. (2004) suggest a comparison of a hybrid zone where selection against hybrids is occurring, to another zone that shows no such selection against hybrids.

For *Mytilus edulis*, hybridization with *M. trossulus* occurs in two geographic regions, the Canadian Maritimes through the eastern Gulf of Maine and in the eastern Atlantic (Scandinavian Baltic Sea). Comparison of these two hybrid zones indicate a bimodal distribution of allele frequencies in the western Atlantic (Bates and Innes 1995; Comesaňa et al. 1999; Rawson et al. 2001), and a unimodal distribution of allele frequencies in the Baltic region (Riginos et al. 2002; Riginos and Cunningham 2005). Following Jiggins and Mallet (2000), the modality of each zone suggests assortative mating and selection against hybrids in the western hybrid zone, and an absence of those same conditions in the east. Levels of gamete compatibility for *M. edulis* and *M. trossulus* in the Baltic hybrid zone remains to be determined, however before the process of reinforcement acting on the blue mussel hybrid zone within the Gulf of Maine can be discounted, a comparison of these two zones should be initiated.

Lemmon et al. (2003) suggest comparing the proportion of heterospecific matings in sympatry, with the expectation that fewer would occur where there is selection against hybridization. The potential for heterospecific matings could be assayed through *in vitro* fertilizations, and actual levels in natural populations through genetic sampling of larvae, or newly settled juveniles, with the expectation that increased gamete incompatibility and fewer heterospecific matings would occur in the eastern Atlantic hybrid zone. The contribution of such a comparison may prove to be invaluable for studying not only the process of reinforcement in the absence of reproductive character displacement, but also lead to a greater understanding of the role of hybridization in species formation and the maintenance of species boundaries in the face of gene flow.

LITERATURE CITED

Arnold, M. L. 1997. Natural Hybridization and Evolution. Oxford Univ. Press, New York, USA.

Arnold, M. L. and S. A. Hodges. 1995. Are natural hybrids fit or unfit relative to their parents? Trends Ecol. Evol. 10: 67-71

Avise, J. C. 1992. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. Oikos 63: 62-76

Babcock R. C., G. D. Bull, P. L. Harrison, A. J. Heyward, and J. K. Oliver. 1986. Synchronous spawning of 105 scleractinian coral species in the Great Barrier Reef. Mar. Biol. 90: 379-394

Babcock, R. 1995. Synchronous multispecific spawning on coral reefs: potential for hybridization and roles of gamete recognition. Rep. Fert. Dev. 7: 943-950

Barton, N. H. and G. M. Hewitt. 1985. Analysis of hybrid zones. Ann. Rev. Ecol. Syst. 16: 113-148

Bates, J. A. and D. J. Innes. 1995. Genetic variation among populations of *Mytilus* spp. in eastern Newfoundland. Mar. Biol. 124: 417-424

Benyon, C. M., D. O. F. Skibinski. 1996. The evolutionary relationship between three species of mussel (*Mytilus*) based on anonymous DNA polymorphism. J. Exp. Mar. Biol. Ecol. 203: 1-10

Bierne, N., P. David, P. Boudry and F. Bonhomme. 2002. Assortative fertilization and selection at larval stage in the mussels *Mytilus edulis* and *M. galloprovincialis*. Evolution 56: 292-298

Blair, W. F. 1964. Isolating mechanisms and interspecies interactions in anuran amphibians. Q. Rev. Biol. 39: 333-344

Blanchard, A. and H. M. Feder. 1997. Reproductive timing and nutritional storage cycles of *Mytilus trossulus* Gould, 1850, in Port Valdez, Alaska, site of a marine oil terminal. Veliger 40: 121-130

Boecklen, W. J. and D. J. Howard. 1997. Genetic analysis of hybrid zones: numbers of markers and power of resolution. Ecology 78: 2611-2616

Brown, W. L. Jr., and E. O. Wilson. 1956. Character displacement. Syst. Zool. 5: 49-64

Butlin, R. 1989. Reinforcement of pre-mating isolation. Pp. 158-179 in *Speciation and its Consequences.* D. Otte and J. A. Endler, eds. Sinauer, Sunderland, MA.

Buss, L. W., and P. O. Yund. 1989. A sibling species group of *Hydractinia* in the northeastern United States. J. Mar. Biol. Assoc. UK 69: 857-874

Byrne, M., and M. J. Anderson. 1994. Hybridization of sympatric *Paitriella* species in New South Wales. Evolution 48: 564-576

Campbell, D., P. Duchesne and L. Bernatchez. 2003. AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. Mol. Ecol. 12: 1979-1991

Coustau, C., F. Renaud and B. Delay. 1991. Genetic characterization of the hybridization between *Mytilus edulis* and *M. galloprovincialis* on the Atlantic coast of France. Mar. Biol. 111: 87-93

Comesana, A. S., J. E. Toro, D. J. Innes and R. J. Thompson. 1999. A molecular approach to the ecology of a mussel (*Mytilus edulis-M. trossulus*) hybrid zone on the east coast of Newfoundland, Canada. Mar. Biol. 133: 213-221

Dobzhansky, T. 1937. *Genetics and the Origin of Species,* Columbia University Press, New York.

_____. 1940. Speciation as a stage in evolutionary divergence. Am. Nat. 74: 312-321

Franke, E. S., R. C. Babcock, and C. A. Styan. 2002. Sexual conflict and polyspermy under sperm-limited conditions: in situ evidence from field simulations with the free-spawning marine echinoid *Evechinus chloroticus*. Am. Nat. 160: 485-496

Freeman K. R., and S. P. MacQuarrie. 1999. Reproduction and pre-settlement behavior of *Mytilus edulis* and *Mytilus trossulus* in controlled environments: implications for mussel culture in mixed-species. Proc. of the Workshop on Mussel Production Capacity (part 2), Canada '98. Bull. Aqua. Assoc. Can. no. 99-3 pp 17-21

Fong, P. 1998. Zebra mussel spawning is induced in low concentrations of putative selective serotonin reuptake inhibitors (SSRIs). Biol. Bull. 194: 143-149

Fukami, H., M. Omori, K. Shimoike, T. Hayashibara, and M. Hatta. 2003. Ecological and genetic aspects of reproductive isolation by different spawning times in Acropora corals. Mar. Biol. 142: 697-684

Gardner, J. P. A. 1996. The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation. Mar. Biol. 125: 385-399

_____. 1997. Hybridization in the sea. Adv. Mar. Biol. 31: 2-78

Gardner, J. P. A. and R. J. Thompson. 2001. The effects of coastal and estuarine conditions on the physiology and survivorship of the mussels *Mytilus edulis, M. trossulus* and their hybrids. J. Exp. Mar. Biol. Ecol. 265: 119-140

Geyer, L. G., and S. R. Palumbi. 2003. Reproductive character displacement and the genetics of gamete recognition in tropical sea urchins. Evolution 57: 1049-1060

Gilg, M. R. and T. J. Hilbish. 2000. The relationship between allele frequency and tidal height in a mussel hybrid zone. Mar. Biol. 137: 371-378

Gosling, E. M. and D. McGrath. 1990. Genetic variability in exposed-shore mussels, *Mytilus* spp. along an environmental gradient. Mar. Biol. 104: 413-418

Grant, W. S., L. Bartlett and F. M. Utter. 1977. Biochemical genetic identification of species and hybrids of the Bering Sea tanner crab, *Chionoecetes bairdi* and *C. opilio*. Proc. Nat. Shell. Assoc. 67: 127

Hagg, W. R. and D. W. Garton. 1992. Synchronous spawning in a recently established population of the Zebra mussel, *Dreissena polymorpha,* in western Lake Erie, USA. Hydrobiologica 234: 103-110

Hagman, D. K., and P. D. Vize. 2003. Mass spawning by two brittle star species, *Ophioderma rubicundum* and *O. squamosissimun.* Science 72: 871-876

Harrison, R. G. 1993. Hybrids and hybrid zones: historical perspective. *in* Hybrid Zones and the Evolutionary Process. R.G. Harrison ed. Oxford Univ. Press, New York, USA. pp – 3-11

Heath, D. D., P. D. Rawson, and T.J. Hilbish. 1995. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Can. J. Fish. Aquat. Sci. 52: 2621-2627

Hellberg, M. E. 1994. Relationships between inferred levels of gene flow and geographic distance in a philopatric coral, *Balanophyllia elegans.* Evolution 48: 1829-1854

Hellberg, M. E. and V. D. Vacquier. 1999. Rapid evolution of fertilization selectivity and lysin cDNA sequences in teguline gastropods. Mol. Biol. Evol. 16:839-848.

Hilbish, T. J., A. Mullinax, S. I. Dolven, A. Meyer, R. K. Kohen and P. D. Rawson. 2000. Origin of the antitropical distribution pattern in marine mussels (*Mytilus* spp.): routes and timing of transequitorial migration. Mar. Biol. 136:69-77

Hilbish, T. J., E. W. Carson, J. R. Plante, L. A. Weaver, and M. R. Gilg. 2002. Distribution of *Mytilus edulis, M. galloprovincialis,* and their hybrids in open-coast populations of mussels in southwestern England. Mar. Biol. 140: 137-142

Howard, D. J. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. p. 46-69. In: R.G. Harrison (ed.), *Hybrid Zones and the Evolutionary Process*. Oxford University Press, New York.

Jiggins, C. D. and J. Mallet. 2002. Bimodal hybrid zones and speciation. Trends. Ecol. Evol. 15: 250-255

Innes, D. J. and J. A. Bates. 1999. Morphological variation of *Mytilus edulis* and *Mytilus trossulus* in eastern Newfoundland. Mar. Biol. 133:691-699

Kirk, H., M. Macel, P. G. L. Klinkhamer, and K. Vrieling. 2004. Natural hybridization between *Senecio jabobaea* and *Senecio aquaticus*: molecular and chemical evidence. Mol. Ecol. 13: 2267-2274

Knowlton, N., J. Mate, H. Guzman, and R. Rowam. 1997. Direct evidence of reproductive isolation among three species of the *Montastrea annularis* complex in Central America (Panama and Honduras). Mar. Biol. 127: 705-711

Kohen, R. K. 1991. The genetics and taxonomy of species in the genus *Mytilus*. Aquaculture 94: 125-145

Kohen, R. K., R. Milkman and J. B. Mitton. 1976. Population genetics of marine pelecypods. IV. Selection , Migration and genetic differentiation in the blue mussel *Mytilus edulis*. Evolution. 30:2-32

Kohen, R. K., J. G. Hall, D. J. Innes, and A. Z. Zera. 1984. Genetic differentiation of *Mytilus edulis* in eastern North America. Mar. Biol. 70: 117-126

Kresge, N., V. D. Vacquier, and C. D. Stout. 2001. Abalone lysin: the dissolving and evolving sperm protein. BioEssays 23: 95-103

Lamare, M. D., and B. G. Stewart. 1998. Mass spawning by the sea urchin *Evechinus chloroticus* (Echinodermata: Echinoidea) in a New Zealand fiord. Mar. Biol. 132: 135-140

Leighton, D. L. and C. A. Lewis. 1982. Experimental hybridization in abalone. Int. J. Invertebe. Reprod. Dev. 5: 273-282

Lemmon, A. R., C. Smadja and M. Kirkpatrick. 2004. Reproductive character displacement is not the only outcome of reinforcement. J. Evol. Biol. 17: 177-183

Lessios, H. A. and C. W. Cunningham. 1990. Gametic incompatibility between species of sea urchin *Echinometra* on the two sides of the Isthmus of Panama. Evolution 44: 933-941

Levitan, D. R. 1998. Does Bateman's principle apply to broadcast-spawning organisms? Egg traits influence in situ fertilization rates among congeneric sea urchins. Evolution 52: 1043-1056.

_____. 2002. The relationship between conspecific fertilization success and reproductive isolation among three congeneric sea urchins. Evolution 56: 1599-1609

_____. 2004. Density-dependant sexual selection in external fertilizers: variances in male and female fertilization success along the continuum from sperm limitation to sexual conflict in the sea urchin *Strongylocentrotus franciscanus.* Am. Nat. 164: 000-000

Levitan, D. R., H. Fukami, J. Jara, D. Kline, T. M. McGovern, K. E. McGee, C. A. Swanson and N. Knowlton. 2004. Mechanisms of reproductive isolation among sympatric broadcast spawning corals of the *Montastrea annularis* species complex. Evolution 58: 308-323

Liou, L. W. and T. D. Price. 1994. Speciation by reinforcement of premating isolation. Evolution 48: 1451-1459

Lopez, J. V., R. Kersanach, S. A. Rehner and N. Knowlton. 1999. Molecular determination of species boundaries in corals: genetic analysis of the *Montastrea annularis* complex using amplified fragment length polymorphisms and a microsatellite marker. Biol. Bull. 196: 80-93

Maddison, W.P. and D.R. Maddison. 2000. *MacClade, Version 4.0.* Sinauer Associates, Inc.

Mallet, A. L. and C. E. Carver. 1995. Comparative growth and survival patterns of *Mytilus trossulus* and *Mytilus edulis* in eastern North America. Mar. Biol. 79: 117-126

Maloy, A., B. J. Barber and P. D. Rawson. 2002. Gametogenesis in a sympatric population of blue mussels, *Mytilus edulis* and *Mytilus trossulus*, from Cobscook Bay, Maine (USA). J. Shell. Res. 22: 119-123

Mardsen, J. R. 1992. Reproductive isolation in two forms of the serpulid polychaete, *Spirobranchus polycerus* (Schmarda) in Barbados. *Bull. Mar. Sci.* 51: 14-18

Marko, P. B. 2002. Fossil calibration of molecular clocks and the divergence times of geminate pairs separated by the Isthmus of Panama. Mol. Biol. Evol. 19: 2005-2021

Marshall, J. L., M. L. Arnold and D. J. Howard. 2002. Reinforcement: the road not taken. *Trends Ecol. Evol.* 17: 558-563

Mayr, E. 1942. *Systematics and the Origin of Species.* Columbia University Press, New York.

McCartney, M. A. and H. A. Lessios. 2002. Quantitative analysis of gametic incompatibility between closely related species of neotropical sea urchins. Biol. Bull. 202: 161-181

McClary, D. J. and M. A. Sewell. 2003. Hybridization in the sea: gametic and developmental constraints on fertilization in sympatric species of *Pseudechinus* (Echinodermata: Echinoidea). J. Exp. Mar. Biol. Ecol. 284: 51-70

McDonald, J. H., R. Seed, and R. K. Koehn. 1991. Allozyme and morphometric characters of three species of *Mytilus* in the northern and southern hemispheres. Mar. Biol. 111: 323-333

Metz, E. C., R. E. Kane, H. Yanagimachi, and S. R. Palumbi. 1994. Fertilization between closely related sea urchins is blocked by incompatibilities during sperm-egg attachment and early stages of fusion. Biol. Bull. 187: 23-34

Minor, J. E., D. R. Fromson, R. J. Britten, and E. H. Davidson. 1991. Comparison of the bindin proteins of *Strongylocentrotus franciscanus, S. purpuratus,* and *Lytechinus variegates*: sequences involved in species-specificity of fertilization. Mol. Biol. Evol. 8: 781-795

Mueller, U.G. and L. L. Wolfenbarger. 1999. AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14: 389-394

Newell, R. I. E., T. J. Hilbish, R. K. Kohen and C. J. Newell. 1982. Temporal variation in the reproductive cycle of *Mytilus edulis* L. (Bivalvia, Mytilidae) from localities on the east coast of the United States. Biol. Bull. 162: 299-310

Palumbi, S. R. 1992. Marine speciation on a small planet. Trends Ecol. Evol. 7: 144-118

_____. 1994. Genetic divergence, reproductive isolation, and marine speciation. Ann. Rev. Ecol. Syst. 25: 547-572

_____. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc. Nat. Acad. Sci. 96: 12632-12637

Palumbi, S. R. and E. Metz. 1991. Strong reproductive isolation between closely related sea urchins (genus *Echinometra*). Molec. Biol. Evol. 8: 227-239

Pawlik, J. 1988. Laval settlement and metamorphosis of sabellariid polychaetes, with special reference to *Phragmatipoma lapidosa*, a reef building species, and *Sabellaria floridensis*, a non-gregarious species. Bull. Mar. Sci. 43:41-60

Pernet, B. 1999. Gamete interactions and genetic differentiation among three sympatric polychaetes. Evolution 53: 435-446
Posada, E. J. and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817-818

Quesada H., C. Gallagher, D. A. G. Skibinski, and D. O. F. Skibinski. 1998. Patterns of polymorphism and gene flow of gender-associated mitochondrial DNA lineages in European mussel populations. Molec. Ecol. 7: 1041-1051

Rawson, P. D. and T. J. Hilbish. 1995. Evolutionary relationships among the male and female mitochondrial DNA lineages in the *Mytilus edulis* species complex. Mol. Biol. Evol. 12: 893-901

_____. 1998. Asymmetric introgression of female and male lineage mitochondrial DNA (mtDNA) haplotypes within a European hybrid zone between *Mytilus edulis* and *Mytilus galloprovincialis*. Evolution 52: 100-108

_____. 1999. Hybridization between the blue mussels *Mytilus galloprovincialis* and *M. trossulus* along the Pacific coast of North America: evidence for limited introgression. Mar. Biol. 134: 201-211

Rawson, P. D., K. Joyner and T. J. Hilbish. 1996. Evidence for intragenetic recombination within a novel genetic marker that distinguishes mussels in the *Mytilus edulis* species complex. Heredity 77:599-607

Rawson, P. D., V. Agrawal, and T. J. Hilbish. 1999. Hybridization between the blue mussels *Mytilus galloprovincialis* and *M. trossulus* along the Pacific coast of North America: evidence for limited introgression. Mar. Biol. 134: 201-211

Rawson, P. D., S. Hayhurst and B. Vanscoyoc. 2001. Species composition of blue mussel populations in the northeastern Gulf of Maine. J. Shell. Res. 1: 31-38

Rawson, P. D., C. Slaughter, and P. O. Yund. 2003. Incomplete gamete compatibility between the blue mussels *Mytilus edulis* and *Mytilus trossulus*. Mar. Biol. 143: 317-325

Riginos, C. and J. H. McDonald. 2003. Positive selection on an acrosomal sperm protein, M7 lysin, in three species of the mussel genus *Mytilus.* Mol. Biol. Evol. 20: 200-207

Riginos, C., and C. W. Cunningham. 2005. Local adaptation and species segregation in two mussel (*Mytilus edulis* x *Mytilus trossulus*) hybrid zones. Molec. Ecol. 14: 381-400

Rignios, C., K. Sukhdeo and C. W. Cunningham. 2002. Evidence for selection at multiple allozyme loci across a mussel hybrid zone. Mol. Biol. Evol. 19:347-351

Riginos, C., M. J. Hickerson, C. M. Henzler and C. W. Cunningham. 2004. Differential patterns of male and female mtDNA exchange across the Atlantic Ocean in the blue mussel *Mytilus edulis.* Evol. 58: 2438-2451

Rundle, H. D., and D. Schluter. 1998. Reinforcement of stickleback mate preferences; sympatry breeds contempt. Evolution 52: 200-208

Saavedra, C., D. T. Stewart, R. R. Stanwood and E. Zouros. 1996. Species specific segregation of gender associated mitochondrial DNA types in an area where two mussel species (*Mytilus edulis* and *M. trossulus*) hybridize. Genetics 143: 1359-1367

Saitou, N. and M. Nei. 1987 The neighbor-joining method: a new method for reconstructing phylogentic trees. Mol. Biol. Evol. 4: 406-425

Sambrook, J. and D. W. Russell. 2000. *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor Laboratory Press

Secor, C. L., A. J. Day and T. J. Hilbish. 2001. Factors influencing differential mortality within a marine mussel (*Mytilus* spp.) hybrid population in southwestern England: reproductive effort and parasitism. Mar. Biol. 138: 731-739

Skibinski, D. O. F., J. A. Beardmore, and T. F. Cross. 1983. Aspects of the population genetics of *Mytilus* (Mytilidae: Mollusca). Brit. J. Linn. Soc. 19:137-183

Slatkin, M. 1991. Interbreeding coefficients and coalescence times. Gen. Res. 58: 167-175

_____. 1993. Isolation by distance in equilibrium and nonequilibrium populations. Evol. 47: 264-279

Sokal, R. R. and F. J. Rohlf. 1995. *Biometry. The Principles and Practice of Statistics in Biological Research. 3rd edition.* W.H. Freeman and Company, New York.
Strathmann, R. R. 1981. On barriers to hybridization between *Strongylocentrotus droebachiensis* (O.F. Müller) and *S. pallidus* (G.O. Sars). J. Exp. Mar. Biol. Ecol. 55: 39-47

Suchanek, T. H., J. B. Geller, B. R. Kreiser and J. B. Mitton. 1997. Zoographic distribution of the sibling species *Mytilus galloprovincialis* and *M. trossulus* (Bivalvia: Mytilidae) and their hybrids in the north Pacific. Biol. Bull 193: 187-194

Swanson, W. J. and V. D. Vacquier. 1998. Concerted evolution in an egg receptor for a rapidly evolving abalone sperm protein. Science 281:710-712

Swofford, D. L. 2001. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Mass.

Takagi, T., A. Nakamura, R. Deguchi and K. Kyozuka. 1994. Isolation, characterization, and primary structure of three major proteins obtained from *Mytilus edulis* sperm. J. Biochem. 116: 598-605

Tamura, K. and M. Nei. 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512-526

Tedengren, B. G., C. Andre, K. Johanneson, and N. Kautsky. 1990. Genotypic and phenotypic differences between Baltic and North Sea populations of *Mytilus edulis* evaluated through reciprocal transplantations. Mar. Ecol. Prog. Ser. 59:221-227

Thompson, J. D., D. G. Higgins and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc. Acids. Res. 22: 4673-4680

Togo, T., K. Osanai, and M. Morisawa. 1995. Existence of three mechanisms for blocking polyspermy in oocytes of the mussel *Mytilus edulis*. Biol. Bull. 189: 330-339

Toro, J., D. J. Innes, and R. J. Thompson. 2004. Genetic variation among life-history stages of mussels in a *Mytilus edulis – M. trossulus* hybrid zone. Mar. Biol. 45: 713-725

Townsend, D. W. 1992. Ecology of larval herring in relation to the oceanography of the Gulf of Maine. J. Plankton Res. 14: 467-493

Vacquier, V. D. 1998. Evolution of gamete recognition proteins. Science 281: 1995-1998

Vainola, R. and M. M. Hvilsom. 1991. Genetic divergence and a hybrid zone between Baltic and North Sea *Mytilus* populations (Mytilidae: Mollusca). J. Linn. Soc. 43: 127-148

Varvio, S. L., R. K. Kohen and R. Vainola. 1988. Evolutionary genetics of the *Mytilus edulis* complex in the north Atlantic region. Mar. Biol. 98: 51-60

Vermeij, G. J. 1991. Anatomy of an invasion: the trans-Artic interchange. Paleobiology 17: 281-307

Wilhelm, R. and T. J. Hilbish. 1998. Assessment of natural selection in a hybrid population of mussels: evaluation of exogenous vs. endogenous selection models. Mar. Biol. 131: 505- 514

Wright, S. 1943. Isolation by distance. Genetics 28: 114-138

Wu, C.-I. 1985. A stochastic simulation study on speciation by sexual selection. Evolution 39: 66-82

Yund, P. O. 1998. The effects of sperm competition on male gain curves in a colonial marine invertebrate. Ecology 79: 328-339.

_____. 2000. How severe is sperm limitation in natural populations of marine free-spawners. Trends Ecol. Evol. 15: 10-13

Yund, P. O., and M. A. McCartney. 1994. Male reproductive success in sessile invertebrates: competition for fertilizations. Ecology 75: 2151-2167

APPENDIX

Appendix A. Modifications to the Glu 5′ PCR amplification protocol (Rawson et al. 1996).

1. PCR amplification conditions: 10.53 mM Tris-HCl, pH 8.3, 52.63 mM KCl, 1.97 mM $MgCl_2$, 2.6 mM dNTP, 1.05 μM Glu 5′ primer, 0.53 μM Ed Glu 5′ and Tross 5 primers, 1.0 units of *AmpliTaq* polymerase (Applied Biosytems (ABI) Foster City, CA)).

2. Thermal cycling conditions were an initial denaturation step of 2 minutes at 95°C, followed by 35 cycles of: 30 seconds at 94°C, 30 seconds at 52°C and 2 minutes at 72°C, with a final extension of 5 minutes at 72°C.

Appendix B. Methods used to calculate sperm concentrations when 50 µl subsamples of the 3rd sperm concentrations fixed in gluteraldehyde were lost to desiccation during transport (2003).

1. A 10 µl subsample of seawater was taken from the fertilization vial with the most concentrated sperm addition (i.e. vial 1 of each cross) and placed on a Neubauer hemacytometer. Sperm numbers were recorded with the pattern of count noted for each grid (Figure 7).

2. The average sperm count and number of squares was calculated and used in the formula: (56)(4000)(1000)(average count)/(average number of squares) where 56 represents the dilution factor, 4000 is the sperm per mm^3, 1000 is a constant to yield the number of sperm in one millimeter of sample [from the Neubauer hemacytometer formula: cells/mL = (dilution)(4,000 squares/mm^3)(1,000 mm^3/cm^3)(cell count)/(number of squares counted)]. This value estimated the number of sperm contained in a 50 µl subsample of the sperm solution used in the first serial dilution (Table 4).

3. This value was then divided by 10 to yield the next, lower, sperm concentration, and repeated for each sperm dilution.

4. In order to estimate the number of sperm the eggs were exposed to during *in vitro* fertilization, the first "small vial" (i.e. sperm concentration in the 50 µl solution) value was divided by 0.46 (the dilution factor calculated from the sperm addition/total volume), and serially divided by 10 as before.

5. Dry sperm was estimated by multiplying the first "egg exposure" value by 10.

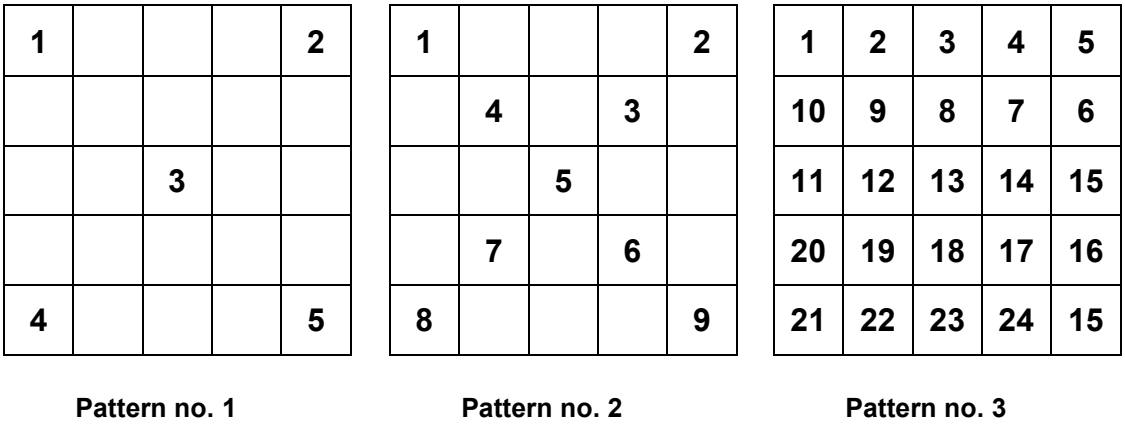


Figure 7. Neubauer hemacytometer patterns for use in calculating sperm concentration.

Table 4. Example of the 2003 sperm concentration calculation.

Neubauer Hemacytometer

Cross	**1** Pattern	Count	Pattern	Count
bC	2	167	2	156
	2 Pattern	Count	Pattern	Count
	2	140	1	120
	3 Pattern	Count	Pattern	Count
	1	134	2	165
			A Average count	**B** Average number of squares
			147	123

Small Vial (sperm concentration in a 50µl subsample)

$$\frac{\mathbf{C}}{(56)(4000)(1000)(\mathbf{A})(\mathbf{B})}$$

1	2	***3**	4	5	6
2.23E+08	2.23E+07	**2.23E+06**	2.23E+05	2.23E+04	2.23E+03

Egg Exposure (sperm concentration under which fertilization occurred)

	C/0.46					
dry sperm	1	2	3	4	5	6
5.84E+09	5.84E+08	5.84E+07	5.84E+06	5.84E+05	5.84E+04	5.84E+03

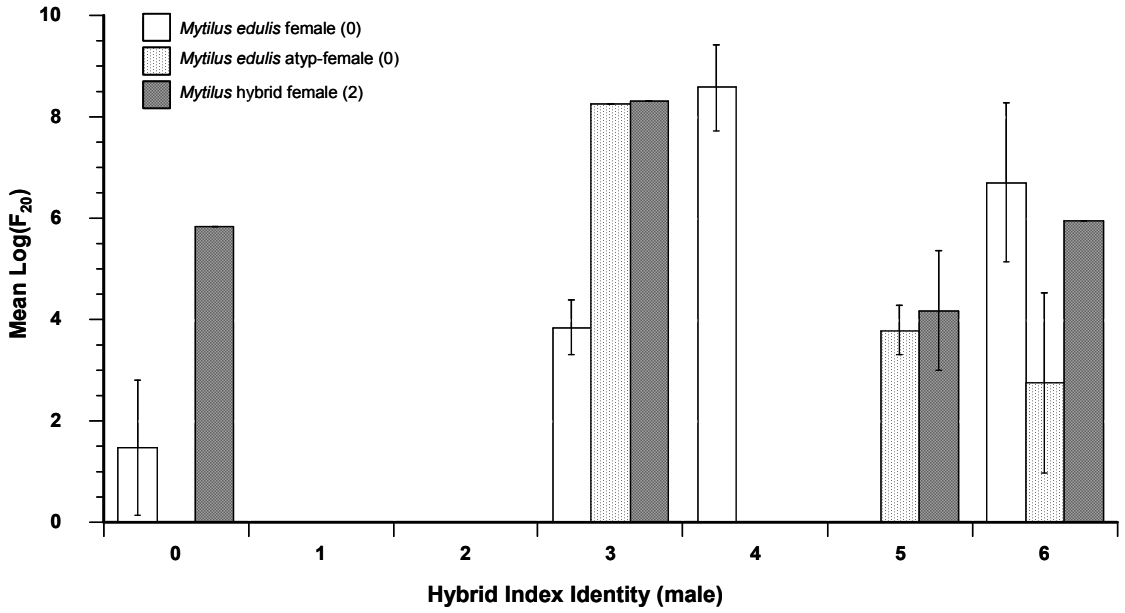
Appendix C. Results from hybrid *in vitro* fertilization experiments.

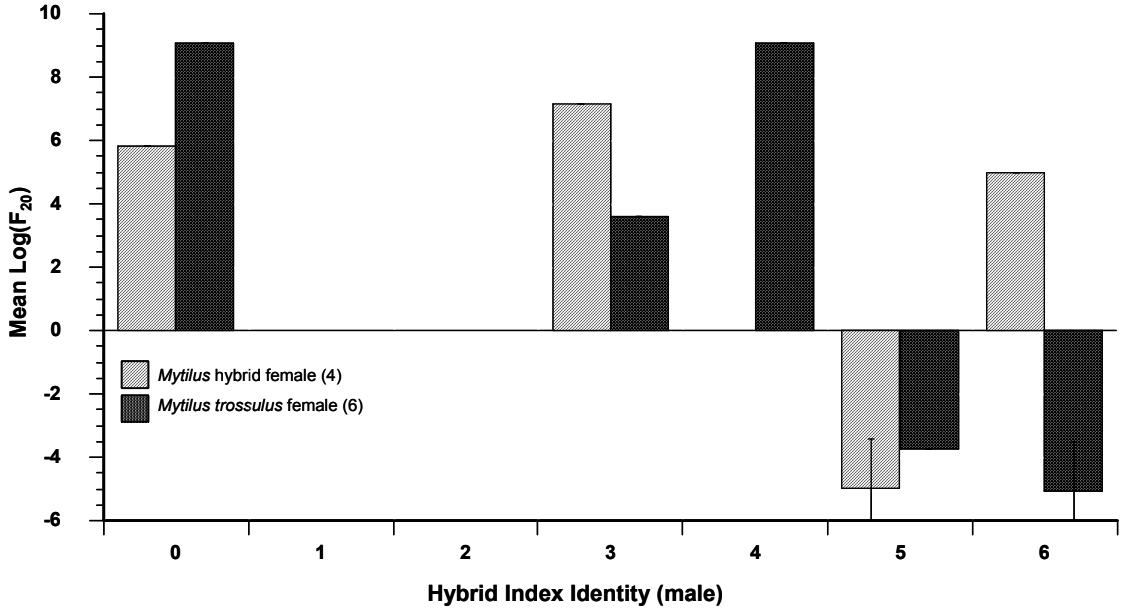
Hybrid individuals were assigned a score based on genetic identity at 3 nuclear loci (*see Chapter 1, Materials and Methods*). An individual with *Mytilus edulis* alleles at each locus receives a score of zero, *M. trossulus* alleles at each locus a score of 6 (1 point each for each *M. trossulus* allele, co-dominant at 3 loci), and hybrids based on number and presence of either a *M. edulis* allele (zero points) or *M. trossulus* allele (1 point). A total of 29 crosses were preformed using hybrid male and female individuals (n = 11, CB; n = 18, K) with scores ranging from 2 (*M. edulis*-like) to 5 (*M. trossulus*-like).

Within both sites, cross type had a significant effect on $\log(F_{20})$ (ANOVA, df_{CB}=6, F=9.30, P<0.001; df_K=3, F=17.02, P<0.001). For sympatric, CB sites, "pure" *Mytilus edulis* females (score 0) showed a signal of assortative mating with respect to crosses involving *M. trossulus* (score 6) and *M. trossulus*-like hybrids (score 4) – having significantly higher $\log(F_{20})$ values when crossed to those males (Tukey post-hoc multiple comparisons, $P_{0x0\text{-}0x6}$<0.0001, $P_{0x0\text{-}0x4}$=0.002)(Figure 8). This pattern of assortative fertilization was less clear in one female that displayed an atypically compatible phenotype (*see Chapter 1, Results*), and in one *Mytilus edulis*-like hybrid (score 2) when crossed to hybrid and *M. trossulus* males. These females showed higher $\log(F_{20})$ when crossed to an F_1 hybrid male (score 3) than to either "pure" species (Figure 7). A *Mytilus trossulus* female and *M. trossulus*-like hybrid female (score 4), were highly compatible with males scored 5 or 6, but less compatible, or strongly blocked, to fertilization from males scored 0-4 (Figure 8).

Females from the K sites also appeared blocked, or strongly blocked to fertilization from males with a score of 3 or higher. Here the mean $\log(F_{20})$ value from crosses involving "pure" *Mytilus edulis* females crossed to "pure" *Mytilus edulis* males was significant different from the mean $\log(F_{20})$ values from all other cross types (Tukey post-hoc multiple comparisons, $P_{0x0\text{-}0x3}$ = 0.005, $P_{0x0\text{-}0x4;0x5;0\text{-}6}$<0.001)(Figure 9).

Figure 8. Patterns of assortative fertilization in *Mytilus edulis*, hybrid, and *M. trossulus* females from Cobscook Bay, ME. Mean log(F_{20}) (±SE) plotted to the hybrid index identity of the male to which the females were crossed. Top plot: *Mytilus edulis* females, score 0; atypically compatible female, score 0; hybrid female, score 2. Bottom plot: hybrid females, score 4; *M. trossulus* female, score 6.


Mytilus edulis female (0)
Mytilus edulis atyp-female (0)
Mytilus hybrid female (2)
Mean Log(F20)
10
8
6
4
2
0
0
1
2
3
4
5
6
Hybrid Index Identity (male)


Mytilus hybrid female (4)
Mytilus trossulus female (6)
Mean Log(F20)
10
8
6
4
2
0
-2
-4
-6
0
1
2
3
4
5
6
Hybrid Index Identity (male)

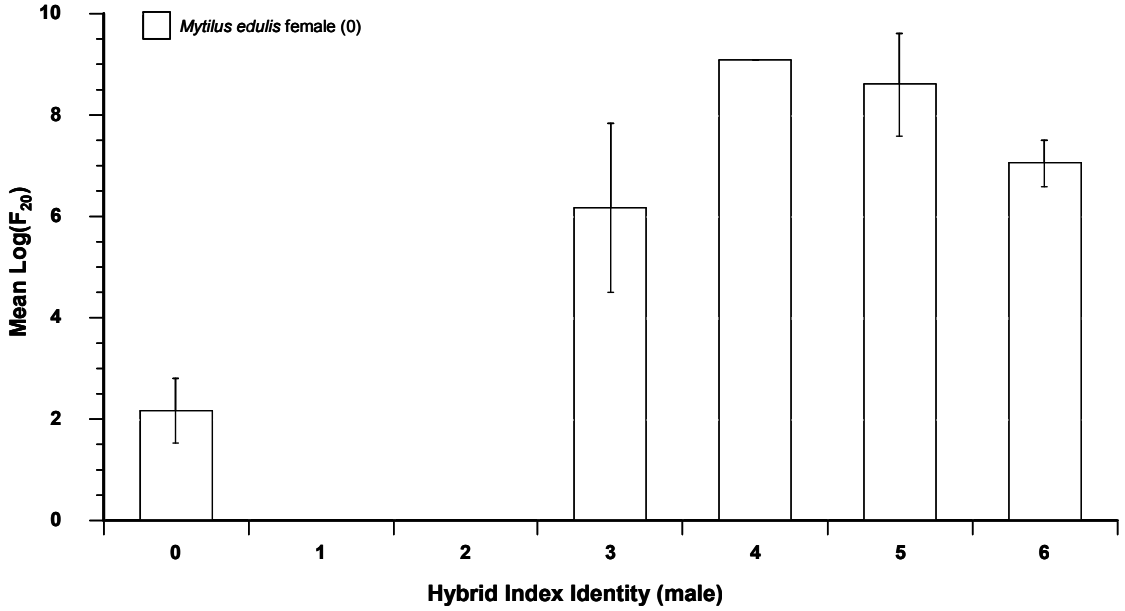


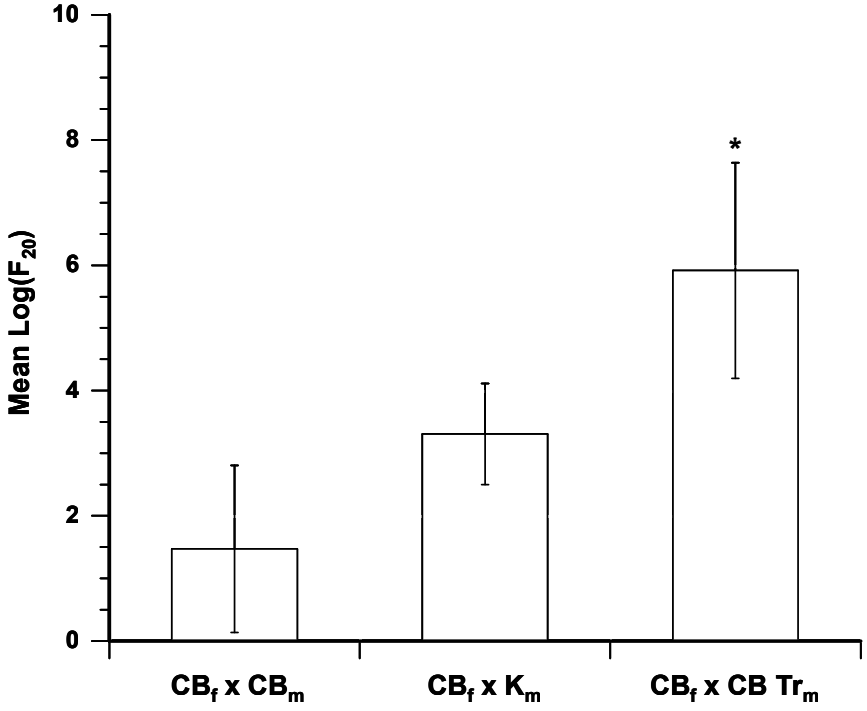
Figure 9. Patterns of assortative fertilization in *Mytilus edulis* females from the allopatric population at Kittery, ME. Mean log(F_{20}) (±SE) plotted to the hybrid index identity of the male to which the females were crossed. Males with hybrid index of >3 were from CB sites, males with score 0, from the K sites.

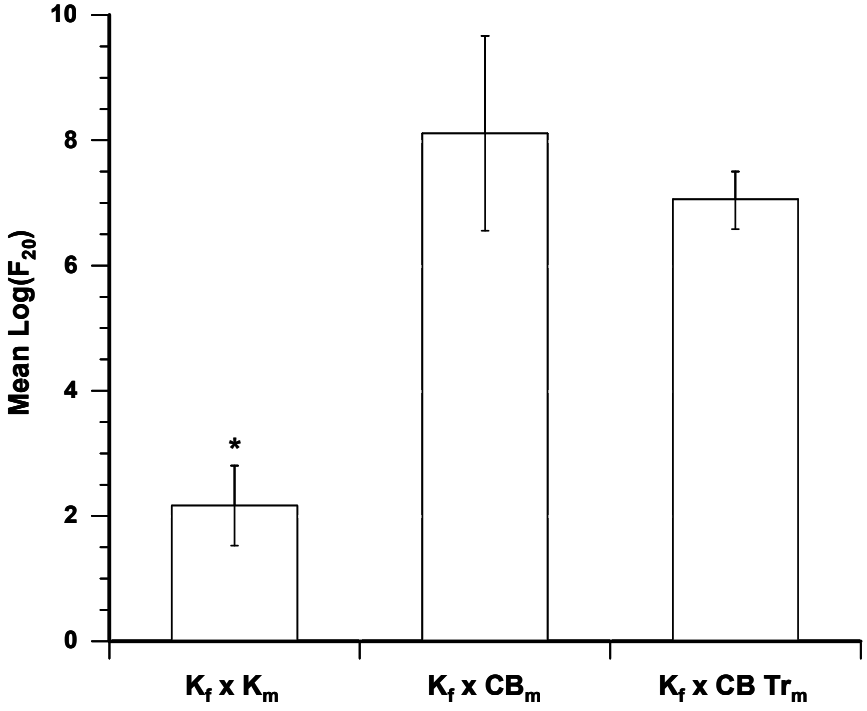
Appendix D. Results from interpopulation *in vitro* fertilization experiments.

Mytilus edulis individuals spawned from the CB and K sites in 2004 were used in interpopulation crosses – here CB site *M. edulis* females (n = 3) were crossed with K site *M. edulis* males (n = 2) , and the reciprocal for K site *M. edulis* females ($n = 4_{Kfemale}$, $n = 2_{CBmale}$). With heterospecific crosses included, crosstype had a significant effect on $\log(F_{20})$ within both sites (ANOVA, df_{CB}=2, F=23.35, P<0.001; df_{K}=2, F=22.77, P<0.001). For CB site females, while the mean heterospecific cross $\log(F_{20})$ was significantly different from mean conspecific $\log(F_{20})$ (Tukey post-hoc multiple comparisons, P<0.001), the mean $\log(F_{20})$ of interpopulation crosses was not. Although, there was a trend of increasing $\log(F_{20})$(Figure 10).

In contrast, within the K site, the mean interpopulation $\log(F_{20})$ was significantly different from the mean conspecific $\log(F_{20})$ (Tukey post-hoc multiple comparisons, P<0.001) – showing K site females to be strongly blocked to fertilization from their own species male, collected from a population sympatric with *Mytilus trossulus*. In this case the mean $\log(F_{20})$ from interpopulation crosses was only marginally significantly different from the mean heterospecific $\log(F_{20})$(Tukey post-hoc multiple comparisons, P=0.55)(Figure 10).

Figure 10. *Mytilus edulis* interpopulation crosses. Mean log(F_{20}) (±SE) plotted to the cross type (*Mytilus edulis* female-site x male site; Tr = *Mytilus trossulus*). Top plot: *Mytilus edulis* females from Cobscook Bay sites; bottom plot: *Mytilus edulis* females from Kittery sites. (*) indicates group mean is significantly different from the others.


10
8
6
4
2
0
Mean Log(F20)
*
CBf x CBm
CBf x Km
CBf x CB Trm


10
8
6
4
2
0
Mean Log(F20)
*
Kf x Km
Kf x CBm
Kf x CB Trm

E. Analysis of geographic differentiation

In order to evaluate a signal of geographic differentiation in *Mytilus edulis,* adult blue mussels (*Mytilus* spp.) used in phylogenetic analysis were collected from six locations: Bras D'Or Lake, Nova Scotia (BO, latitude 45° 45′N; longitude 60° 45′W), Cobscook Bay, ME (CB, latitude 44°52′N; longitude 67°07′W); Kittery, Maine (K, latitude 43°04′N; longitude 70°41′W); Narragansett Bay, Rhode Island (RI, latitude 44° 25′N; longitude 71° 27′W); Stony Brook Harbor, New York (LIS, latitude 40° 55′; longitude 73° 9′W); and the Pacific coast of North America (PCT) (Figure 11). Both the Bras D'Or Lake and Cobscook Bay samples were obtained from mixed populations of *Mytilus edulis* and *M. trossulus*, while the remaining samples were taken from putatively allopatric populations of *M. edulis* (except the Pacific coast, allopatric *M. trossulus*).

Tissue used in DNA extractions was obtained either from a biopsy clipped from the mantle frill, or from adductor muscle. Genomic DNA was extracted using a modification of the "Rapid Isolation of Mammalian DNA" protocol (Sambrook and Russell 2000), and individuals were identified to species using three nuclear DNA PCR-based markers that are diagnostic for *Mytilus edulis* and *M. trossulus*; Glu 5' (Rawson et al. 1996; modifications Appendix I), ITS (Heath et al. 1995) and Mal I (Rawson et al.
2001). Only individuals identified as "pure" species (i.e. having the same species-specific identity for each locus) were used in subsequent analysis.

Sequences generated were edited using Sequencher (ver. 4.1.1, Gene Codes Corp.) and aligned using Clustal *X* software (Thompson et al. 1994). Sequence errors and stop codons, and identical sequences were checked using MacClade 4.0 (Maddison and Maddison 2000). F-mtDNA (Figure 12) and M-mtDNA (Figure 13) phylogenetic trees were constructed with PAUP* 4.0 (Swofford 2001) using neighbor-joining (NJ) (Saitou and Nei 1987) and maximum likelihood (ML) based on the Tamura-Nei (Tamura and Nei 1993) model with gamma distribution of rates and variable sites (TrN+G) for F-COI and M-COI (Arlequin 2.0.1.1)); determined to be the best-fitting models of molecular evolution from hierarchical likelihood ratio tests preformed in Modeltest 3.6 (Posada and Crandall 1998).

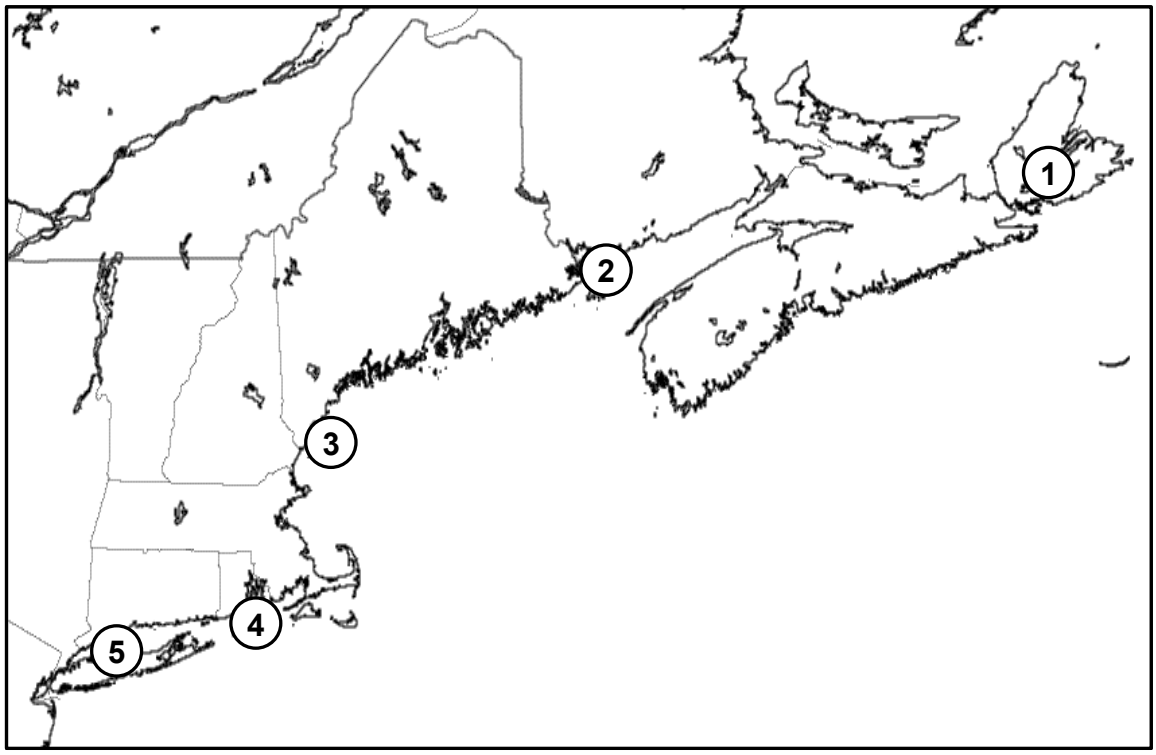


Figure 11. Collection sites for *Mytilus edulis* and *M. trossulus* used in phylogeographic analysis. Site 1: Bras D'Or Lake, Nova Scotia; site 2: Cobscook Bay, ME; site 3: Kittery, Maine; site 4: Narragansett Bay, Rhode Island; site 5: Stony Brook Harbor, New York. Not pictured, Pacific coast collection site of "PCT" *M. trossulus*.

Figure 12. *Mytilus* spp. F-mtDNA (COI) gene genealogy. Neighbor-joining tree based on maximum likelihood distances calculated under the TrN+G model of evolution, bootstrapped in 1000 replicates. Individuals labeled with site of collection as identified in text and Figure 6. CB site individuals identified by label used for *in vitro* fertilization assays (B20, B82, W25, Y55, Y14); K site 2003 individuals identified as "WB-." Nodes labeled with an asterisk (*) indicate a weak bootstrap support of 50%. Identical haplotypes are grouped as a single sequence, but listed individually.

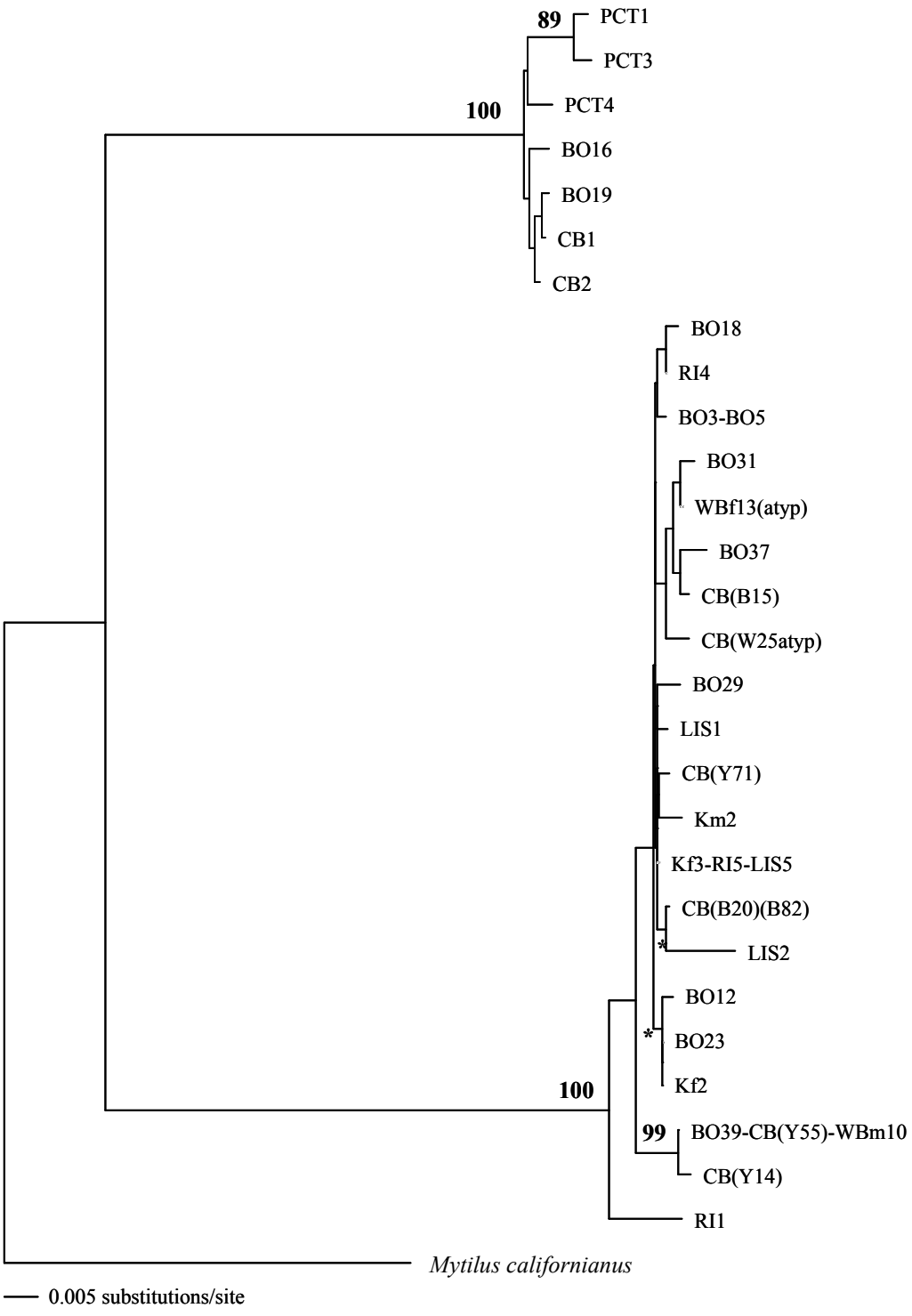
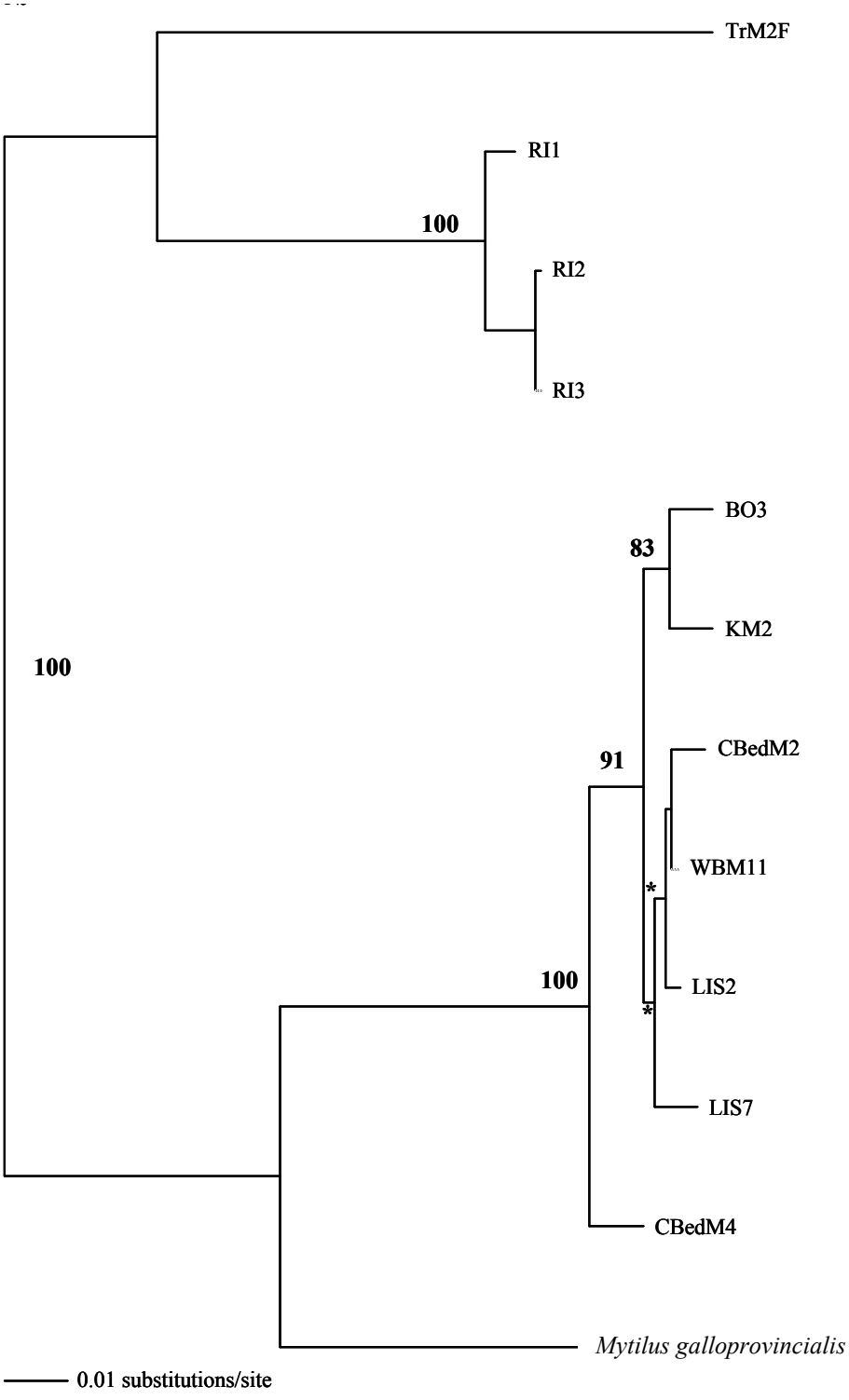

89
PCT1
PCT3
100
PCT4
BO16
BO19
CB1
CB2
BO18
RI4
BO3-BO5
BO31
WBf13(atyp)
BO37
CB(B15)
CB(W25atyp)
BO29
LIS1
CB(Y71)
Km2
Kf3-RI5-LIS5
CB(B20)(B82)
*
LIS2
BO12
*
BO23
Kf2
100
99
BO39-CB(Y55)-WBm10
CB(Y14)
RI1
Mytilus californianus
0.005 substitutions/site

Figure 13. *Mytilus* spp. M-mtDNA (COI) gene genealogy. Neighbor-joining tree based on maximum likelihood distances calculated under the TrN+G model of evolution, bootstrapped in 1000 replicates. Individuals are labeled with site of collection as identified in text and Figure 6, TrM2 is from the CB site. Nodes labeled with an asterisk (*) indicate a weak bootstrap support of 50%.


TrM2F
RI1
100
RI2
RI3
BO3
83
KM2
100
CBedM2
91
WBM11
*
100
LIS2
*
LIS7
CBedM4
Mytilus galloprovincialis
0.01 substitutions/site

For the ML search, 10 random-sequence stepwise additions were used for starting trees with TBR branch swapping (100 replicates). Support for nodes in the NJ and ML trees were obtained using a full heuristic search bootstrap procedure (NJ 1000 replicates, ML 100 replicates). The outgroup for the F-mtDNA consisted of a single sequence from *Mytilus californianus* (GenBank #U73812), M-mtDNA trees were midpoint rooted but included a single sequence from *M. galloprovincialis* (GenBank #AY363687).

F. AFLP analysis of *Mytilus* spp. genome

The utility of Amplified Fragment Length Polymophisms (AFLP™) analysis was investigated as a means of providing a higher resolution of hybrid zone modality and level of introgression in sympatric *Mytilus edulis* and atypically compatible *M. edulis* females. A total of 21 primer combinations (Applied Biosystems) were examined for *Mytilus* species-specific fragments (Table 5). A species-specific peak was defined as a peak being fixed and present in a "pure species" individual (scored 0 for *Mytilus edulis* or 6 for *M. trossulus* from PCR based genetic assays) while absent in all individuals of the alternate "pure species".

At the onset of the investigation, it was determined that *Mytilus* DNA restricts with EcoRI and MseI restriction enzymes, yielding a product smear between 100-1500 bp. In order to achieve a minimum of approximately 70-100 detected peaks, where a loss of peaks occurred at larger fragment size, modifications to the AFLP™ Plant Mapping Protocol (1997) included, (1) the use of high molecular weight (>3000 bp) crude genomic DNA, (2) cleaning of the genomic DNA (Qiagen column, eluted in water), and (3) 1 in 3 dilution of preselective amplification product (i.e. 10 μl preselective amplification product, 20 μl $TE_{0.1}$ pH 8.0). Peaks were detected using GeneScan Analysis software (v. 3.7) with a threshold amplitude set to 50, fragment size defined as ± 0.5 bp, under a size standard of GS 500-250, and an analysis parameter set to GS500; peak detection parameters recommended based on use of an ABI 3100 Sequencer.

Using the above criteria, analysis using Genotyper (v. 3.7 NT), and examination by eye, a total of 15 peaks (at 8 of the 21 primer combinations) were defined as being species-specific; 7 that appeared present and fixed in *Mytilus edulis*, and 8 in *M. trossulus* individuals. Fragment size ranged from 164-418 bp in individuals with a score of 0 (*M. edulis,* n=3) and 52-489 bp in individuals with a score of 6 (*M. trossulus* n=3). The majority of species-specific peaks had a fragment size range of 200-300 bp. Later analysis, using a larger sample size of both *Mytilus edulis* and *M. trossulus* resulted in the loss of species-specificity in all but one peak, with one primer combination (P10, 264 bp, fixed-present in *M. edulis*, fixed-absent in *M. trossulus*).

Table 5. Primer combinations used in AFLP™ analysis of *Mytilus edulis*-*M. trossulus* hybrid zone. Mean (± SD) number of peaks per species listed. Primer combinations without values indicate a failed combination, generating < 10 peaks in both species.

Primer Code	EcoR I	Mse I	*Mytilus edulis*	*Mytilus trossulus*
P1	ACT	CAA	114	86
P2	ACT	CAC	73 (17)	76 (5)
P3	ACT	CAG	64 (12)	62 (21)
P5	ACT	CTA	74 (14)	76 (21)
P7	ACT	CTG	61 (17)	78 (15)
P9	ACA	CAA	92 (17)	99 (4)
P10	ACA	CAC	78 (31)	78 (15)
P12	ACA	CAT	86 (4)	92 (20)
P14	ACA	CTC	89 (21)	66 (19)
P17	AAC	CAA	-	-
P18	AAC	CAC	-	-
P24	AAC	CTT	-	-
P25	ACC	CAA	-	-
P33	AGC	CAA	-	-
P41	AAG	CAA	104 (10)	105 (11)
P42	AAG	CAC	64 (4)	87 (9)
P44	AAG	CAT	98 (4)	83 (6)
P49	AGG	CAA	88 (17)	83 (6)
P51	AGG	CAG	-	-
P57	ACG	CAA	51 (17)	57 (5)
P61	ACG	CTA	62 (21)	66 (23)